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DISSERTATION

Expression patterns of EphA2 tyrosine kinase receptor in human digestive system organs and tumors

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Abstract

Introduction

Early diagnosis and targeted therapy are important for successful treatment of cancer.

Therefore, antibodies and peptides are more and more utilized as theranostics in cancer targeted imaging and therapy. They can directly induce therapeutic effects after binding the corresponding molecules in malignant tumors. Furthermore, specific peptides and antibodies can be conjugated with pharmaceuticals or diagnostic agents to enable targeted therapy and diagnosis.

The ephrin type-A receptor 2 (EphA2) plays important roles both in tumorigenesis and in angiogenesis. This receptor has attracted considerable attention as a promising target for cancer detection and treatments. Before EphA2 receptor-targeting antibodies or peptides are effectively used as selective agents, it is necessary to analyze what the exact expression pattern of EphA2 receptor is in malignant tumors and normal tissues.

Method

Here, the expression pattern of EphA2 was analyzed in eight different normal tissues and corresponding carcinomas of the digestive system, using immunohistochemical staining.

Results

Similar intensity of EphA2-expression was shown in tissue sections of stomach, cardia adenocarcinoma, cholangioadenocarcinoma and hepatocellular carcinoma, compared to their corresponding adjacent normal tissues, respectively.

In particular, esophageal squamous cell carcinoma displayed strong expression of EphA2, however this expression was reduced when compared to normal esophagus tissue. Although EphA2 was found in normal esophagus, the esophageal squamous cell carcinoma displayed the strongest expression of EphA2 compared to cardia, stomach, colorectal, pancreatic adenocarcinoma and hepatocellular carcinoma.

Remarkably, EphA2 was overexpressed in three adenocarcinomas, including esophageal, cardia and pancreatic adenocarcinoma, compared to adjacent normal tissues. Furthermore, the EphA2 receptors were differentially localized in these three carcinomas. In esophageal and cardia adenocarcinoma EphA2 was overexpressed at the intracellular part of malignant cells, whereas in pancreatic adenocarcinoma the plasma membrane highly expressed EphA2.

Finally, the data show that EphA2 expression did not correlate with the pathologic differentiation in any of the eight carcinomas.

Discussion

In conclusion, the data suggest that EphA2 receptor is a target structure in esophageal, cardia and pancreatic adenocarinoma and suitable for further analysis of EphA2 receptor-binding molecules in these three carcinomas. Based on this study it is proposed to explore future targeting agents dependent on the cellular localization of EphA2. Since the EphA2 receptor is strongly expressed in esophageal squamous cell carcinomas compared to non-squamous cell carcinomas, it is suggested to expand the analyses of EphA2 to other organs, particularly those having high incidences of squamous cell carcinoma formation.

Zusammenfassung

Einleitung

Das frühzeitige Erkennen und die gezielte Therapie sind entscheidend für die erfolgreiche Behandlung von Krebserkrankungen. Dazu finden Antikörper und Peptide als Theranostika immer häufiger Anwendung in der zielgerichteten Bildgebung und Therapie. Diese können direkte therapeutische Effekte durch Bindung an Zielstrukturen maligner Tumoren induzieren. Weiterhin können spezifische Peptide oder Antikörper an Pharmazeutika oder diagnostische Agenzien gekoppelt werden, um eine zielgerichtete Diagnose und Therapie für Krebserkrankungen zu ermöglichen.

Der Ephrin Typ-A Rezeptor 2 (EphA2) spielt wichtige Rollen in der Karzinogenese und Angiogenese. Dieser Rezeptor ist von besonderem Interesse, da er eine vielversprechende Zielstruktur für die Detektion und Behandlung von Krebserkrankungen darstellt. Bevor aber gegen EphA2 Rezeptoren gerichtet Peptide oder Antikörper als selektive Agenzien zum Einsatz kommen, ist es wichtig exakte Expressionsmuster von EphA2 in maligenen Tumoren und in Normalgeweben zu bestimmen.

Methode

Das Expressionsmuster des EphA2 Rezeptors wurde im Normalgewebe sowie in maligenen Tumoren, acht verschiedener Organe des Verdauungsapparates, durch immunhistochemische Färbungen untersucht.

Ergebnisse

Die Analyse des EphA2 Rezeptors in Gewebeschnitten von Magen-, Kardia-, und Cholangiokarzinomen sowie hepatozellulären Karzinomen zeigte eine unveränderte Expression verglichen mit anliegendem Normalgewebe.

Insbesondere im Plattenepithelkarzinom des Ösophagus wurde eine starke Expression des EphA2 Rezeptors festgestellt, die jedoch im Vergleich zum gesunden Ösophagus geringer ausfiel. Obwohl EphA2 im Ösophagus detektiert wurde, zeigten korrespondierende Plattenepithelkarziome die stärkste Expression verglichen mit Kardiakarzinomen, Magen-karzinomen, kolorektalen und pankretischen Karzinomen als auch mit hepatozellulären Karzinomen.

Hervorzuheben ist, dass die Überexpression des EphA2 Rezeptors in Adenokarzinomen des Ösophagus und des Pankreas sowie in Kardiakarzinomen, im Vergleich zum Normalgewebe detektiert wurde. Darüber hinaus waren die EphA2 Rezeptoren in diesen drei Adenokarzinomen unterschiedlich lokalisert. So wurde EphA2 in Kardiakarzinomen und ösophagialen Adenokarzinomen vor allem intrazellulär expremiert, wohingegen in pankreatischen Adenokarzinomen EphA2 in der Plasmamembran detektiert wurde.

Schliesslich konnte gezeigt werden, dass die Expression des EphA2 Rezeptors in allen acht untersuchten Karzinomen nicht mit der pathologischen Differenzierung der Tumorer-krankungen assoziiert war.

Schlussfolgerung

Diese Daten lassen darauf schließen, dass der EphA2 Rezeptor eine Zielstruktur für ösopagilae und pankreatische Adenokarzinome sowie Kardiakarzinome ist und sich für weitere Analysen EphA2-bindender Moleküle eignet. Darauf basierend sollten weitere zielgerichtete Agenzien erforscht werden, die abhängig von der zellulären Lokalisation von EphA2 eingesetzt werden können. Da die stärkste EphA2-Expression im Plattenepithel-karzinom des Ösophagus, verglichen mit anderen Karzinomen detektiert wurde, sollte die Analyse von EphA2 auch auf andere Organe ausgeweitet werden, insbesondere Organe, welche hohe Inzidenzen zur Bildung von Plattenepithelkarzinomen haben.

1. Introduction

1.1 Background

1.1.1 Global cancer statistics

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [1]. Due to demographic changes of an aging population and increasing adoption of cancer-causing behaviours, the global burden of cancer is continuously rising. In 2008, about 12.7 million cancer cases and 7.6 million cancer deaths occurred worldwide, and the number of deaths from cancer is projected to rise to over 13.1 million also in the next two decades [2]. Accordingly, improved diagnostic, therapeutic and preventive strategies are needed in order to reduce the incidence and the mortality rate of this disease.

1.1.2 Status of current cancer treatments

A diverse group of treatments can be implemented to manage the disease of cancer, among which, the most widely utilized nowadays in the clinic regime are surgery, chemotherapy and radiotherapy [3, 4]. Surgery, besides its curative effects that can be either the removal of only the tumor or the entire organ, is often necessary for the evaluation of the exact tumor extent. Chemotherapy, in contrast with surgery, is regarded as a systemic therapeutic pathway and applied by oncologists to shrink the cancer, to relieve the symptoms, or to prolong life by inducing remission. It is usually restricted to a series of cytotoxic drugs which produce anticancer effects by destroying rapidly dividing cancer cells in the body using the mechanism of disrupting the duplication of DNA or mitotic structures of the splitting cells. Unfortunately, due to the non-targeted mode of action, these conventional chemotherapeutic drugs also attack dividing cells in normal organs, especially those tissues having a high replacement rate, e.g. skin, bone marrow, hair follicles, epithelium of gastrointestinal tract, and subsequently result in a great deal of side effects, which often lead to fatal lesions in patients who are initially supposed to survive the cancer temporarily. Radiotherapy, terminologically refers to a therapeutic approach which uses high-energy radiation to shrink tumors and kill cancer cells by interfering with the DNA directly or creating charged particles within the cells that can in turn damage the DNA. Radiation may be delivered either by a machine outside the body or a radioactive material placed in the body near cancer cells. Depending on the same mechanistic factor of interfering with dividing cells as in chemotherapy, radiotherapy also destroys normal cells and causes severe acute and chronic side effects, some of which are even result in permanent damage.

1.2 Targeted cancer therapy and imaging ─ a promising cancer management

Considering the circumstance that conventional chemotherapy and radiotherapy result in high systemic toxicity and adverse side effects, how would it be possible to gain more specific management of the cancer? Targeted cancer therapy and imaging, by selectively focusing on molecular and cellular changes that are specific to cancer, is addressing this problem and increasingly becoming state-of-the-art in the therapeutic and diagnostic fields of cancer.

Paul Ehrlich, the famous biologist and immunologist, whose concept of a “magic bullet” (that drugs are able to go straight to their intended cell structural targets specifically with high affinity) is the first original version of description of targeted therapy, and is credited as the milestone in the field of cancer therapy development [5]. Following Ehrlich’s ingenious insights, and with the exceptional advances in molecular biology and genetic research over the past decades, anticancer development has begun to move away from traditional cytostatic agents that result in systemic toxicity and adverse side effects to the current exploration of “personalized and tailored” targeted molecules that block growth and spread of cancer by precisely targeting the specific molecules involved in tumor growth and progression.

1.2.1 Monoclonal antibody and antibody-drug conjugates ─ the pioneer for targeted therapy

Monoclonal antibodies that became the first available entity in the field of targeted therapy emerged in the late 1990s, and marked an impressive step towards the realization of the magic bullet concept. Mainly, they function as suppressors to negatively regulate tumor growth and progression by specifically binding to the target cells or proteins that are crucial for the pathogenesis of cancer [6]. So far, a range of monoclonal antibodies have been approved by US Food and Drug Administration (FDA) for treating a diversity of cancer diseases. Examples include the anti-HER2 antibody trastuzumab used in breast cancer, the anti-CD20 antibody rituximab used in a variety of B-cell malignancies, and the anti-VEGF-A antibody bevacizumab used in colorectal cancer [7-9]. However, because of problems associated with their immunogenic potential and rather high molecular mass, these fascinating anticancer candidates did not prove to be very inspiring in their development in the clinic [10, 11].

In the last 10 years, great development of antibody engineering technology has enabled cancer targeting concepts to be transformed into antibody-drug conjugates [12]. This new type of targeted compound, which consists of an antibody (or antibody fragment such as a single-chain variable fragment) linked to a payload agent such as toxins, cytokines or radionuclides, has proved a promising approach for treating cancer patients both in therapy and in diagnostic imaging [13-17]. Current antibody conjugates predominately target lymphohaematopoietic disease owing to accessibility, as illustrated by gemtuzumab ozogamicin and anti-CD22 immunotoxin BL22. For the treatment of solid tumors, the association of geldanamycin with the therapeutic monoclonal antibody trastuzumab showed enhanced efficacy in a murine xenograft tumor model compared with trastuzumab alone [18-20].

1.2.2 Radionuclide labeled somatostatin peptide analogs — an example for effective peptide-based receptor-targeted tumor imaging and therapy

In the mid-1980s tumors were recognized to overexpress peptide hormone receptors on the surface of their cells [21]. This discovery was soon applied to generate alternative targeting conjugates comprised of a signaling molecule (a radionuclide) or therapeutic agent (a toxin) linked to a peptide receptor ligand. A pioneer of this model are conjugates of somatostatin analogs (octreotide, lanreotide, and vapreotide) labeled with radionuclides for diagnostic imaging of gastroenteropancreatic neuroendocrine tumors known to express high density of somatostatin receptors [22]. By *in vivo* somatostatin receptor scintigraphy using intravenous injection of the radiolabeled somatostatin analogs, neuroendocrine tumors and their metastases can be precisely localized. This sensitive procedure is superior to all standard diagnostic tools available today for the detection of specific neuroendocrine tumors [23]. At the therapeutic level, several pilot studies using high doses of somatostatin analogs radiolabeled with ^{90}Y or somatostatin analogs coupled to potent cytotoxic agents have shown a great inhibition or at least a stabilization of the tumor growth [24-28].

The successful introduction of radiolabeled synthetic analogs of somatostatin for the detection of gastroenteropancreatic neuroendocrine tumors and their metastasis in the clinic was a major breakthrough in the field of receptor-targeted tumor imaging and therapy. It offered a brand new concept to design the modality of targeting compounds for molecular diagnostic imaging and therapy of cancer.

1.3 Peptides, novel agents for receptor-targeted tumor imaging and therapy

Peptides are molecules consisting of several amino acids linked together with peptide bonds. The size of peptides can vary from molecules with only two amino acids to as many as 80 or more amino acids. They generally do not only exist in natural form (e.g. somatostatin) but also can be designed synthetically as novel molecules. As tumor-homing molecules, unlike monoclonal antibodies limited with excessive molecular mass alongwith high immunogenicity and low permeabilization in tissue, the nature of peptide itself, in particular its molecular structure and behavior, makes it an attractive molecular entity to act as a therapeutic or delivery agent to target corresponding receptors *(Table 1)* [29].

Advantages	**Disadvantages**
Small size	Rapidly degraded by peptidases
Excellent permeability in tissue	
No or low antigenicity	
Minimal side effects	
Easy to synthesize and modify chemically	
Easy to link to chelators	
Easy to radiolabel	
High-affinity receptor binding	
Rapid clearance from the body	
No brain targeting due to inability to cross the blood-brain barrier	

***Table 1*: Comparison of advantages and disadvantages of peptide as a selective agent for targeting strategies as compared with antibodies**

1.4 Hepatocellular receptor tyrosine kinase class A2 (EphA2) ─ a potential target for molecular diagnostic imaging and therapy of cancer

Receptor tyrosine kinases (RTKs) are high-affinity cell surface receptors for many polypeptides, e.g. growth factors, cytokines, and hormones. Of the 90 unique tyrosine kinase genes identified in the human genome, 58 encode receptor tyrosine kinase proteins. It has been shown that RTKs are key regulators not only for physiological processes in normal cells but also in the progression of many types of cancer [30]. While several tyrosine kinase receptors have been documented and targeted for their critical roles in tumorigenesis, the cell membrane bound ephrin type-A receptor 2 (EphA2), a member of Eph tyrosine kinases receptor family, has generated great interest in recent years [31].

1.4.1 Structure of EphA2 receptor

EphA2 was first identified in 1990 as a result of screening an epithelial cell cDNA library with degenerate oligonucleotides designed to hybridize to highly conserved regions of protein tyrosine kinases [32]. It was initially referred to as eck (epithelial cell kinase) for its expression in the majority of epithelial cells. The human EphA2 gene which is located on chromosome 1p36, encodes a receptor tyrosine kinase of 976 amino acids with an apparent molecular weight of 130 kDa and has a 90 % amino acid sequence homology to mouse EphA2 [33]. The EphA2 extracellular regions include an N-terminal ligand binding domain, a cysteine-rich motif, and two fibronectin-like repeats. Intracellularly, the juxtamembrane domain contains two conserved tyrosines that undergo autophosphorylation and is followed by a tyrosine kinase domain. The COOH-terminal end of EphA2 serves as a docking site for interacting proteins that may mediate downstream single transduction processes and includes a sterile α motif and a PDZ protein domain-binding motif [34].

Five ligands have been demonstrated for EphA2 ─ EphrinA1 to EphrinA5. The most extensively studied is EphrinA1, which is a glycosylphosphatidylinositol (GPI) anchored protein with an apparent molecular mass of 28 kDa [35]. Before EphrinA1 was identified as the ligand for EphA2, it was discovered as a novel tumor necrosis factor-α inducible gene product in human umbilical vein endothelial cells [36].

1.4.2 EphA2-EphrinA1 signaling and associated elements

EphA2-EphrinA1 signaling functions like the classical tyrosine kinase receptor-mediated cell signaling wherein a cell bearing an EphA2 receptor, upon binding to EphrinA1 ligand, transmits signals downstream known as forward signaling. Similarly, intracellular signals in backward direction are also generated in EphrinA1-bearing cells which is known as reverse signaling [37]. As both EphA2 and EphrinA1 are membrane bound, their interaction occurs only at the sites of cell-cell contact. The contact between two communicating cells and a clustering of membrane bound EphrinA1 ligand in the opposite cells are required for the EphA2-EphrinA1 bidirectional signaling [34, 38].

Typically, upon the binding of EphrinA1 in a “lock and key” mechanism, EphA2 receptor becomes tyrosine phosphorylated and interacts with several adapter proteins to elicit downstream signaling transduction [34, 39]. The key downstream molecules of this signaling are phosphatidyl inositol 3’ kinases (PI3K), Src family kinases, Rho and Rac1 GTPase, mitogen activated protein kinases (MAPK) and integrins along with the crosstalk of other oncogenic receptors (e.g. epidermal growth factor receptor, EGFR) that regulate cell adhesion, proliferation, migration, and modulation of cytoskeleton architecture, and development of vascular network [40]. However, there is a unique characteristic that makes EphA2 different from most RTKs in that ligand binding and phosphorylation of the conserved tyrosine may not be necessary for the kinase activity of EphA2 [41, 42]. In fact, EphA2 may possess ligand-independent kinase activity in tumor cells and trigger EphrinA1 independent signaling in malignancy in a non-tyrosine-phosphorylated state [41, 43]. In addition, EphA2 participates in feedback loops that may switch between different outputs depending on the state of other cellular signaling networks. This makes the signaling pathway of EphA2 extremely complex [44]. Examples include EphA2─H-Ras─Erk negative feedback loop: Activation of the H-Ras─Erk pathway increases EphA2 expression through Mek1 and decreases EphrinA1 expression. In turn, EphrinA1-dependent EphA2 activation inhibits H-Ras─Erk signaling and also downregulates EphA2 levels by causing receptor internalization and degradation; EphA2─E-cadherin positive feedback loop: E-cadherin expression increases EphA2 expression, surface localization, interaction with EphrinA1 and consequently forward signaling. In turn, EphA2 signaling enhances E-cadherin-mediated adhesion.

To date, little is known about EphrinA1 reverse signaling.

1.4.3 The expression and function of EphA2 in physiology and cancer

There is much evidence showing that Eph-Ephrin signaling mediates crucial functions in normal developmental tissues. In the nervous system, it regulates the development of neuronal networks, axon guidance, formation and remodeling of synaptic connections, and nervous system repair [44]. In the gastrointestinal tract, it controls the positioning process along the crypt-villus axis of intestinal cells, as well as the maintenance of stem cells and the proliferation of progenitor cells in the intestine [45]. It has also been shown that Eph-Ephrin signaling plays an essential role in the morphogenesis of the vascular system during embryonic development [46, 47].

Interestingly, EphrinA1-dependent EphA2 forward signaling was also thought to be detrimental for tumor progression. Initial studies showed that forcing EphA2 receptor activation with soluble EphrinA1 Fc fusion proteins can inhibit proliferation, survival, and migration and invasion of many types of cancer cells in culture as well as tumor growth in several mouse models [48-50]. Subsequent experiments further indicated that EphA2 receptor activated by EphrinA1 acquires the remarkable ability to inhibit oncogenic signaling pathways, such as H-Ras─Erk, PI3K─Akt and Abl─Crk pathways [44].

Although EphrinA1-dependent EphA2 forward signaling induces inhibitory effects in tumorigenesis, this inhibition is often difficult to realize in most situations in malignant cells. Because of the function loss of E-cadherin, malignant cells often exhibit a decreased cell adhesion, which would prevent neighboring cells expressing receptors and ligands from productively interacting with each other [41]. This phenomenon directly results in decreased ligand-induced receptor activation, internalization and degradation, and contributes to the stability and overexpression of EphA2 in malignant cells [51]. This explanation is consistent with the observation from several previous studies that EphA2 is presented at much low levels, tyrosine phosphorylated, and localized at the sites of cell-cell contact of non-neoplastic epithelia, but abundantly overexpressed, non-tyrosine-phosphorylated, and settled at membrane-ruffles in aggressive cancer cells [52]. Depending on its overexpression and kinase activity in the absence of EphrinA1, EphA2 is able to interact with adaptor proteins and triggers EphrinA1 independent signaling pathways that are most intimately involved in the pathogenesis of cancer [53-56].

Multiple cell models and clinical specimens have reported that EphA2 receptor was frequently elevated and appears to be an active participant in malignant progression in various type of cancer, including breast cancer, aggressive melanomas, ovarian cancer, non-small cell lung cancer, prostate cancer, and colon cancer[42, 57-61]. It has been also shown in many cancer cell lines that the highly expressed EphA2 receptors are poorly activated by EphrinA1, as judged by their low level of tyrosine phosphorylation [42, 55, 62]. A vast number of other studies show that the mere presence of EphA2 was able to elicit oncogenic effects. EphA2 overexpression caused oncogenic transformation of mammary epithelial cells in culture as well as *in vivo* [42, 63]. Downregulation of EphA2 by small interfering RNAs (siRNAs) or antisense oligonucleotides decreased cancer cell malignancy in culture and inhibited tumor growth in several mouse cancer models[43, 64, 65]. EphA2 overexpression induced and sustained phosphorylation and kinase activity of FAK in prostate and pancreatic cancer cells and therefore increased the expression of matrix metalloproteinase-2, an integral player in tumor cell invasion [66-68]. EphA2 was found to be required for Src-dependent invasiveness of colorectal cancer cells in culture [69]. Activation of RHOA downstream of EphA2 promoted ameboid-type migration of cancer cells and destabilized epithelial adherent junctions in various cancer cell lines [70, 71]. EphA2-ERBB2 crosstalk activated the H-Ras─Erk pathway and RHOA in a mouse mammary tumor model, enhancing tumor growth and cell proliferation and migration [54, 72].

1.4.4 The expression and function of EphA2 in tumor microenvironment

Aside from the function of EphA2 signaling in tumorigenesis, several investigations addressed the importance of the EphA2-EphrinA1 interaction specifically in the context of tumor angiogenesis, both *in vitro* and *in vivo*. Initial studies found that EphA2 was specifically expressed at the endothelial cells of breast carcinoma and Kaposi’s sarcoma xenografts in a tyrosine-phosphorylated state [73]. Subsequent investigation exhibited that EphrinA1 could stimulate EphA2-expressing endothelial cell migration, sprouting and survival, and such a process could be inhibited by administration of recombinant EphA2-Fc [74]. Furthermore, use of EphA2-Fc as a blocker of the receptor-ligand interaction revealed that disruption of this process *in vivo* inhibited angiogenesis and tumor growth in several animal models [75, 76]. The importance of EphA2 in angiogenesis and metastasis was even more evident in the finding that metastatic mammary carcinoma cells implanted into EphA2-deficient mice exhibited decreased tumor volume and metastasis [77]. By contrast, studies using transplantable mouse mammary tumor model expressing native tumor-cell produced EphrinA1 also showed a proangiogenic effect through induction of EphA2-specific endothelial cell migration and increasing vascular endothelial growth factor expression. Moreover, siRNA knockdown of EphrinA1 in mammary tumor cells inhibited angiogenesis and lung metastasis [78].

Taken together, these findings show that, depending on cell types and whether there is an EphrinA1 ligand or not, the EphA2 receptor functions in drastically different ways. Generally, in tumor cells, it was found to promote tumorigenesis independently of the EphrinA1 ligand, but oppositely inhibit tumor progression after being activated and phosphorylated by EphrinA1 ligand. In endothelial cells within tumor microenvironment, it was shown to be able to positively regulate tumor neovascularization depending on EphrinA1 ligand and therefore participated in tumor angiogenesis.

1.4.5 Available EphA2 receptor-dependent targeted therapeutics for malignancy

The function of EphA2 receptor is quite complex, but without a doubt it contributes to both tumorigenesis and angiogenesis. Due to its overexpression in many different types of cancer and to its important role in pathogenesis of cancer, targeting EphA2 receptor has become a very attractive approach for cancer therapy. To date, a number of agents based on interfering with the tumor-promoting effects of EphA2 have been developed. Downregulation of EphA2 gene expression by using siRNAs or antisense oligonucleotides has been shown to be able to inhibit malignant cell behavior both *in vitro* and *in vivo* [43, 64, 79]. By mimicking the actions of EphrinA1 ligand, several agonistic monoclonal antibodies against EphA2 exhibited a potent capability to decrease the proliferation and migration of various cancer cells, and the level of tumor growth and metastasis in mouse models [49, 80-82]. A bispecific antibody engineered to simultaneously bind EphA2 and the T cell receptor-CD3 complex has also shown to effectively promote regression of EphA2 expressing tumor cells [83]. In addition, drugs, toxins or other substances have been conjugated to agonistic monoclonal antibodies to target tumors expressing high levels of EphA2. 1C1-mcMMAF, the EphA2 monoclonal antibody (1C1), coupled to monomethyl auristin phenylalanine (MMAF) via a non-cleavable linker maleimidocaproyl, was able to reduce EphA2 expression in a time-dependent manner in cancer cells in culture, and cause tumor destruction in orthotopic ovarian cancer model [84]. In the meantime, the 1C1 antibody was also utilized to label with copper molecules (^{64}Cu) to develop an EphA2 specific non-invasive imaging technology [85].

2. Goal of the thesis

Current cancer therapy relies heavily on indiscriminate, highly toxic, chemotherapeutic agents resulting in systemic toxicity and adverse side effects. An ideal solution to such limitations would be the selective delivery of anticancer drugs to cancer tissues.

EphA2 is a member of receptor tyrosine kinases, playing an important role in both tumorigenesis and angiogenesis. Therefore, it is being actively studied as a potential target for tumor diagnosis and treatment.

To this end, a number of strategies have been developed to target EphA2 receptor, among them, the monoclonal antibody-based therapeutics are demonstrated high frequently. However, while these candidates can display high affinity and tumor specificity, they suffer from clinical limitations. For example, the formulation and preparation of homogeneous antibody-drug conjugates faces challenges due to protein instability, and the risk of overwhelming immune responses from the host. In this regard, peptides, featuring its numerous advantages, show a great potential to overcome those limitations, and represent the most attractive molecules as the therapeutic or delivery agent for receptor-targeted cancer therapy and imaging.

To gain a tumour-homing therapeutic or diagnostic agent, a very important molecular basis for the EphA2 receptor-targeted tumour management is the presence of EphA2 in a high incidence and intensity in carcinomas of interest, but a zero or low incidence in normal tissues. In order to offer the EphA2-targeting research a supportive molecular backup, it is necessary to acquire the knowledge about what kind of EphA2 expression difference there is between normal and carcinoma tissues express.

The goal of this thesis was to investigate the expression pattern of EphA2 receptor in digestive system organs and their corresponding carcinomas.

3. Materials and methods

3.1 Materials

3.1.1 Snap frozen tissues

Snap frozen specimens from various organs of human digestive system were obtained from the department of “Klinik für Allgemein-, Viszeral- und Transplantationschirurgie, Campus-Virchow-Klinikum, Charité”.

Tissue type	Case number
Esophageal squamous cell carcinoma	11
Esophageal and cardia adenocarcinoma	9
Adjacent normal esophagus	4
Stomach adenocarcinoma	6
Adjacent normal stomach	3
Colon adenocarcinoma	10
Adjacent normal colon	6
Pancreatic adenocarcinoma	7
Adjacent normal pancreas	3
Cholangiocarcinoma	17
Hepatocellular carcinoma	17
Adjacent normal liver	3

3.1.2 Formalin fixed paraffin embedded tissues

Multiple formalin fixed paraffin embedded tissue micro arrays were purchased from companies.

Tissue type	Case number	Company source
Esophageal squamous cell carcinoma	30	AccuMax
Adjacent normal esophagus	34	
Esophageal adenocarcinoma	9	US Biomax
Adjacent normal esophagus	7	
Cardia adenocarcinoma	22	US Biomax
Adjacent normal cardia	5	
Stomach adenocarcinoma	24	AccuMax
Adjacent normal stomach	8	US Biomax
Colon adenocarcinoma	45	AccuMax
Adjacent normal colon	3	
Pancreatic adenocarcinoma	27	AccuMax
Adjacent normal pancreas	26	
Cholangiocarcinoma	46	AccuMax
Adjacent normal liver	3	
Hepatocellular carcinoma (with B viral history)	49	AccuMax
Adjacent normal liver	2	

3.1.3 Antibodies and protein marker

<u>Primary antibody</u>

Polyclonal goat anti human EphA2 antigen affinity-purified IgG was purchased from R and D system, Germany.

Polyclonal normal goat IgG isotype control was purchased from R and D system, Germany.

<u>Secondary antibody</u>

Polyclonal rabbit anti goat biotinylated, affinity-isolated IgG was purchased from Dako Cytomation, Denmark.

Polyclonal horseradish peroxidase-conjugated affinity-purified donkey anti goat IgG(H+L) was purchased from Dianova, Germany.

<u>Protein Marker</u>

PageRuler^{TM} Plus Prestained Protein Ladder was purchased from Fermentas, European Union.

3.1.4 Chemicals and reagents

Methanol	Merck, Germany
Acetone	Merck, Germany
Xylene	Carl Roth, Germany
Isopropanol	Merck, Germany
Ethanol	Herbeta Arzneimittel, Germany
Sodium chloride	Merck, Germany
Di-sodium hydrogen phosphate dihydrate	Carl Roth, Germany
Potassium chloride	Carl Roth, Germany
Potassium dihydrogen phosphate	Merck, Germany
Citric acid monohydrate	Merck, Germany
Triton X-100	Calbiochem, USA
Rabbit serum	PAA, Austria
Bovine serum albumin	Sigma-Aldrich, USA
30 % Hydrogen peroxide	Merck, Germany
Hematoxylin	Dako, Germany
Glycerol	Merck, Germany

Tris(hydroxymethyl)aminomethane	Carl Roth, Germany
Hydrochloric acid	Merck, Germany
Sodium dodecyl sulfate	Carl Roth, Germany
Protease inhibitor cocktail tablet	Roche, Germany
Bromphenol blue	Merck, Germany
Dithiothreitol	SERVA, Germany
Ammonium Persulfate	Sigma-Aldrich, USA
Tricine	Carl Roth, Germany
Polyacrylamide (Acrylamide/Bisacrylamide 29:1)	Carl Roth, Germany
N,N,N',N'-Tetramethylethylenediamine, 1,2-Bis(dimethylamino)-ethane	Sigma-Aldrich, USA
Glycine	SERVA, Germany
96 % Ethanol denatured with methyl ethyl ketone	Merck, Germany
Brilliant blue G250	Carl Roth, Germany
Acetic acid	Merck, Germany
Ponseau S	Sigma-Aldrich, USA
Tween 20	Merck, Germany
Non fat milk powder (blotting grade)	Carl Roth, Germany

3.1.5 Solutions and buffers

PBS	137 mM NaCl PH 7.4 2.68 mM KCl 8 mM Na_2HPO4 1.8 mM KH_2PO4
BSA/PBS	137 mM NaCl 2.68 mM KCl 8 mM Na_2HPO4 1.8 mM KH_2PO4 0.1 % (w/v) BSA

Citrate buffer (PH6.0)	10 mM Citric acid monohydrate
H_2O_2 /PBS	90 % (v/v) PBS
	10 % (v/v) H_2O_2
H_2O_2 /methanol/PBS	30 % (v/v) PBS
	60 % (v/v) methanol
	10 % (v/v) H_2O_2
Triton X-100/PBS	99.9 % (v/v) PBS
	0.1 % (v/v) Triton X-100
Lysis buffer	100 mM Tris pH8.8
	1 % (w/v) SDS
Laemmli buffer	50 mM Tris pH6.8
	1 % (w/v) SDS
	10 % (v/v) Glycerol
	0.02 % (w/v) Bromphenol blue
	100 mM DTT
Top buffer	100 mM Tris
	100 mM Tricine
	0.1 % (w/v) SDS
	do not adjust pH (PH≈8.25)
Bottom buffer	200 mM Tris pH8.8
Transferring buffer	192 mM Glycine
	25 mM Tris
Ponceau S solution	0.1 % (w/v) Ponceau S
	5 % (v/v) acetic acid

PBST	137 mM NaCl pH7.4 2.68 mM KCl 8 mM Na$_2$HPO4 1.8 mM KH$_2$PO4 0.1 % (v/v) Tween 20
Milk-PBST	137 mM NaCl 2.68 mM KCl 8 mM Na$_2$HPO4 1.8 mM KH$_2$PO4 0.1 % (v/v) Tween 20 5 % (w/v) non-fat milk powder
Coomassie-staining solution	0.1 % (w/v) Coomassie Brilliant blue G250 50 % (v/v) Methanol 7.5 % (v/v) Acetic Acid
Fixing solution	25 % (v/v) Isopropanol 10 % (v/v) Acetic acid

3.1.6 Gel solution recipes

8 % resolving gel

Solution components	**1 Gel (ml)**	**2 Gels (ml)**	**4 Gels (ml)**
ddH$_2$O	2.3	4.6	9.3
30 % acrylamide mix	1.3	2.7	5.3
1.5 M Tris pH 8.8	1.3	2.5	5.0
10 % SDS	0.05	0.1	0.2
10 % APS	0.05	0.1	0.2
TEMED	0.006	0.009	0.018

5 % stacking gel

Solution components	**2 Gels (ml)**	**4 Gels (ml)**
ddH$_2$O	3.4	6.8
30 % acrylamide mix	0.83	1.66
1 M Tris pH 6.8	0.63	1.26
10 % SDS	0.05	0.1

10 % APS	0.05	0.1
TEMED	0.007	0.01

3.1.7 Kits

Biotin blocking system	Dako, Denmark
Avidin biotin complex kit	Vector Laboratories, USA
Liquid DAB+substrate chromogen system	Dako, Denmark
Pierce BCA protein assay reagent A and B	Thermo Scientific, USA
Super signal west dura extended duration substrate	Thermo Scientific, USA

3.1.8 Instruments

Fume hood

Rocking table

Vortexer

Electrophoresis apparatus

Blotting equipments

Microscope

Spectrophotometer

99° C incubator

Pressure cooker

4° C extra-speed centrifuge

Glassware

Gel equipments

Developing machine

Graphic analysis software

Gel scanner

Toast oven

3.2 Methods

3.2.1 Immunohistochemical staining of snap frozen tissues

The presence of EphA2 in snap frozen specimens was analyzed on the protein level by avidin biotin complex immunohistochemical staining detection system. All incubations were carried out in a humidified chamber to avoid drying of the tissues. For fixation, ice-cold methanol and acetone were mixed for a fixative in ratio of 1:1. Tissue sections were swiftly immersed in the fixative for 2 min and afterwards air dried. The fixated sections were washed twice with 1×PBS and then immersed in H_2O_2 /PBS solution for 10 min to quench endogenous peroxidise activity, followed by two times 1×PBS washing again. To block endogenous biotin, sections were incubated with unconjugated avidin for 20 min and then with unconjugated biotin for 20 min to block the residual biotin-binding activity of avidin. Sections were then washed two times with

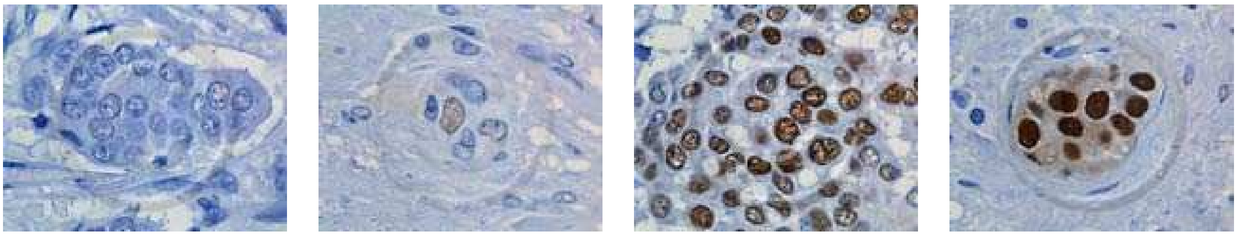
1×PBS and treated with 0.1 %Triton X-100/PBS for 10 min to enhance the penetration of antibody. Nonspecific antigen was masked on sections by 5 % rabbit serum in PBS for 30 min before incubating overnight at 4° C with goat anti human-EphA2 polyclonal primary antibody diluted in 0.1 % BSA/PBS solution (1:300). Normal goat IgG was set to replace the primary antibody for an isotype control. Sections were then washed four times with 1×PBS and incubated with biotinylated rabbit anti goat polyclonal secondary antibody diluted in PBS (1:300) for 30 min. For the preparation of avidin biotin complex (ABC) reagent, one drop of reagent A and one drop of reagent B were added to 5 ml 1×PBS in a mixing bottle, and the mixture was allowed to stand for 30 min before use. Sections were washed with 1×PBS twice and incubated with ABC reagent for 30 min. Following two washings with 1x PBS, sections were covered with DAB solution for 30 sec to develop a brown colour at the site of antigen on the tissue. The DAB incubation was stopped by deionized water and sections were afterwards counterstained with hematoxylin for 10 min. Counterstaining was stopped using tap water and finally sections were mounted with glycerol gelatine using coverslips.

3.2.2 Immunohistochemical staining of formalin fixed paraffin embedded tissues

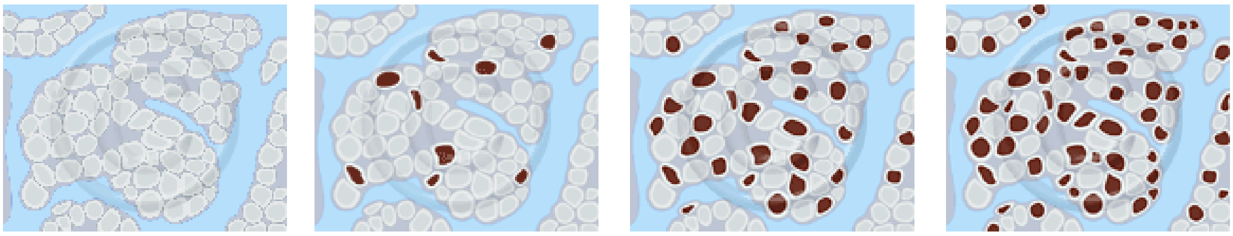
The expression pattern of EphA2 in formalin fixed paraffin embedded tissue micro array specimens was analyzed by avidin biotin complex immunohistochemical staining detection system. As frozen specimens, all incubations were performed in a humidified condition to protect specimens from drying. Tissue micro array sections were first toasted in an oven for 20 min at 65° C, then they were deparaffinized in xylene for 15 min twice and isopropanol for 5 min, afterwards, rehydrated in 96 %, 80 %, 70 % and 50 % ethanol for 5 min, respectively. Following washing with 1×PBS twice, the antigen retrieval was processed in 1×citrate buffer for 2 min to expose antigen on the sections by means of a pressure cooker. Sections were then washed twice with 1×PBS and immersed in H_2O_2 /methanol solution for 5 min, followed by another 5 min 1×PBS washing. After 10 min incubation in 0.1 % Triton X-100/PBS and 5 min washing with 1×PBS, sections were blocked by 5 % rabbit serum in PBS for 30 min before incubating overnight at 4° C with goat anti human-EphA2 polyclonal primary antibody diluted in 0.1 % BSA/PBS solution (1:150). Normal goat IgG was set to replace the primary antibody for an isotype control. The following procedure was monitored the same as frozen sections in above, except that the time for DAB incubation was 5 min instead.

3.2.3 Semi-quantitative evaluation of EphA2 expression level under microscope

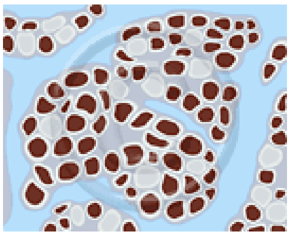
The EphA2 expression was reflected by immunoreactive score (IRS) which was analyzed using a semi-quantitative scoring system recommended by Remmele and Stegner in 1987 [86]. Based on this system, staining intensity (SI) and the positive percentage (PP) of epithelial cells were the two factors used to calculate the IRS by adapting the formula of $IRS = SI \times PP \times 100$. The IRS values were classified into three possibilities of range: $5 < IRS \leq 10$, $10 < IRS \leq 100$ and $100 < IRS$. These three ranges were then correspondingly transformed into plus sign of +, ++ and +++ to finally represent the positive expression level. The EphA2 expression was regarded as negative when $IRS \leq 5$ *(Fig. 1 and Table 2).*



(SI) 0 = No staining (SI) 1 = Weak staining (SI) 2 = Moderate staining (SI) 3 = Strong staining



$PP \leq 1\ \%$ $1\ \% < PP \leq 10\ \%$ $10\ \% < PP \leq 50\ \%$ $50\ \% < PP \leq 80\ \%$



$80\ \% < PP$

***Figure 1*: Representative figures for evaluating staining intensity and the positive area in tumor cells** [86] **.** The intensity of staining was scored 0, 1, 2 and 3. The positive area was measured within the range of 0 % ~ 100 %.

PP \ SI	No staining (0 point)	Weak staining (1 point)	Moderate staining (2 points)	Strong staining (3 points)
PP = 0 %	IRS = 0	IRS = 0	IRS = 0	IRS = 0
0 < PP ≤ 1 %	IRS = 0	IRS = 0 ~ 1	IRS = 0 ~ 2	IRS = 0 ~ 3
1 % < PP ≤ 10 %	IRS = 0	IRS = 1 ~ 10	IRS = 2 ~ 20	IRS = 3 ~ 30
10 % < PP ≤ 50 %	IRS = 0	IRS = 10 ~ 50	IRS = 20 ~ 100	IRS = 30 ~ 150
50 % < PP ≤ 80 %	IRS = 0	IRS = 50 ~ 80	IRS = 100 ~ 160	IRS = 150 ~ 240
80 % < PP	IRS = 0	IRS > 80	IRS > 160	IRS > 240

***Table 2*: IRS values calculated by the formula of IRS = SI (the point's value)× PP × 100.** Depending on this formula, score of 0 and 300 are the minimum and maximum values for IRS, respectively.

3.2.4 Extracting protein off tissue sections

A denaturing lysis buffer for extracting whole cell protein was prepared as mentioned above. An appropriate ready-to-use cocktail tablet of protein inhibitor was freshly added to the lysis buffer. All extracting steps were processed on ice unless otherwise mentioned. For a 20 µm thick, 1×1 cm scale piece of tissue mounted on a slide, 50 µl lysis buffer was added. Tissues were homogenized with a tip directly on the slide for 3 min, and then kept at room temperature for 2 min. The tissue lysate was then aspirated and transferred into an Eppendorf tube which was prefilled with a filtered tip, followed by centrifuging at 13000 rpm for 3 min at 4° C. Finally, the filter was discarded and the lysate was stored at - 80° C.

3.2.5 Protein determination

The protein concentration of tissue lysates was determined using the thermo scientific pierce BCA protein assay kit. High purified BSA was selected as a reference standard each time the assay was performed, and was prepared with a storage concentration of 10 mg/ml in the same lysis buffer as the protein being extracted. BCA protein assay reagents A and B were mixed in a proportion of 50:1 for 1 min at room temperature, a dilution-series of BSA standards were then prepared by diluting it in the reagent mixture to10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.313 mg/ml and 0.156 mg/ml. Protein samples were diluted in the same reagent mixture in proportions of 1:2, 1:4, 1:8. 100 µl of each above differentially diluted BSA standards and protein samples were loaded onto a 96-well plate as instructions, and was incubated at 37°C for 30 min. In the presence of protein, the light green reagent mixture was changed to purple. The absorbance values were measured using a spectrophotometer at 562 nm. The responses of the BSA standards were used to calculate a standard curve. Absorbance values of protein samples were then interpolated onto the formula for the standard curve to determine their concentrations. All probes were run in duplicates.

3.2.6 Coomassie staining

To ensure that each lane is loaded with an equal amount of protein, coomassie staining was used in every western blot for the protein normalization. After SDS-PAGE, the gel was rinsed briefly in dH2O and then incubated for 3 min in fixing solution, for 30 min in coomassie-staining solution, and again for 3 min in fixing solution. Finally, gel was destained in dH2O until faint bands were clearly visible.

3.2.7 Western Blot

Expression of EphA2 in normal and tumor snap frozen specimens was measured by Western Blot. The resolving and stacking gels were compounded as mentioned in material. First, the gel chamber was assembled as manufactory instructed. Then, the resolving gel was poured into the chamber and covered with isopropanol for 30 min to allow polymerization. Later on, the isopropanol was discarded and the stacking gel was poured instead, afterwards and rapidly, a 10-well-comb was inserted into the stacking gel. The whole gel was then kept at room temperature for another 30 min for polymerization. For the analysis, 5 μg protein from each sample was loaded onto the gel. An equal volume of 2×Laemmli buffer was added to the samples (1:1), followed by incubation for 5 min at 99° C for protein denaturing. Samples were then mixed, centrifuged briefly at 3000 rpm for 30 sec, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a condition of 100 V for 10 min for the stacking and 180 V for 90 min for the resolving. Approximate locating of proteins on SDS-PAGE by a ready-to-use prestained protein ladder (10 kDa~250 kDa). To accomplish the transfer, a frozen Bio-Ice cooling unit and a standard stir bar were added in the transfer tank to help maintain low and even buffer temperature and ion distribution. Under a condition of 110 V for 90 min, the previously electrophoresed proteins on the SDS-PAGE were transferred to a nitrocellulose membrane through a gel sandwich that was built up in accordance with instructions. The membrane was then blocked in 5 % milk-PBST solution for 30 min at room temperature before incubating overnight at 4° C with goat anti human-EphA2 polyclonal antibody in blocking solution (1:500). The membrane was washed four times with 1×PBST followed by addition of horseradish peroxidase-conjugated donkey anti goat polyclonal antibody in blocking solution (1:4000) for 1 h at room temperature. The membrane was then washed four times with 1×PBST. Antigen-antibody reactions were visualized using an enhanced chemiluminescence (ECL) system according to the manufacturer's instructions by means of X-ray films.

3.3 Statistical analysis

Chi-square test and Fisher's exact test were used to assess the statistical significance of the cellular localization of EphA2 between normal and corresponding tumor tissues, of the EphA2 expression rate between normal and corresponding tumor tissues, and among eight types of tumor tissue as well as among three histological differentiations of each single type of tumor tissue. Partitions of Chi-square method was used for the pair-wise comparisons of rates. Wilcoxon rank sum test was applied for evaluating the statistical significance of the expression level of EphA2 between normal and corresponding tumor tissues. Kruskal-Wallis H test was utilized to assess the statistical significance of the expression level of EphA2 among eight types of tumor tissue, and among three histological differentiations of each single type of tumor tissue. Nemenyi test was used for the pair-wise comparisons of rank. The results were considered significant when the *P* value was less than 0.05. All statistical procedures were performed with SPSS software package (Version 19.0).

4. Results

4.1 Validation of the R and D manufactured primary antibody

In order to acquire an exact expression pattern of a tentative target, a reliable and specific antibody is quite important. As we were dealing with a polyclonal antibody, it was necessary to ensure that this antibody is specific enough for its antigen (target). Therefore, for the first step, we collected snap frozen tissues from seven kinds of organs, and monitored them by immunohistochemical staining and subsequent western blot so as to confirm the reliability and specificity of the antibody.

4.1.1 Immunohistochemical staining showed the specificity of the antibody

Seven pairs of normal and carcinoma snap frozen tissues were monitored by immunohistochemical staining (IHCs). The IHCs results showed that the R and D manufactured primary antibody was able to recognize the plasma membrane anchored and intracellular localized EphA2 with high affinity and ideal specificity, which was reflected not only by the definitive expression and localization of EphA2 in target cells, but also by the negative outcomes in negative and isotype controls, where the primary antibodies were respectively replaced by PBS and isotype antibody instead. EphA2 was positively detected in diverse manner in the following tissues: normal esophagus (4/4), esophageal squamous cell carcinoma (9/11), esophageal adenocarcinoma (8/9), normal stomach (2/3), stomach adenocarcinoma (6/6), normal colon-rectum (3/6), colorectal adenocarcinoma (6/10), normal pancreas (1/3), pancreatic adenocarcinoma (6/7), normal bile duct (3/3), cholangiocarcinoma (5/17), normal liver (2/3) and hepatocellular carcinoma (3/17). All pictures are showed in the appendix.

4.1.2 Western blot further confirmed the reliability of the antibody

Given the results from IHCs, we picked out three representatives presenting either positive or negative EphA2 expression from each type of paired normal and carcinoma tissue, and monitored them by western blot, using the same primary antibody that we used for IHCs. Furthermore, the results of western blot convinced us that the results from IHCs were reliable, since that samples holding positive EphA2 expression in immunohistochemistry consistently showed positive targeting bands in western blot at the sites of correct molecular weight of 130 kDa *(Fig. 2, Fig. 3)*, whereas samples negatively expressing EphA2 in IHCs exhibited no band in western blot *(Fig. 4, Fig. 5)*.

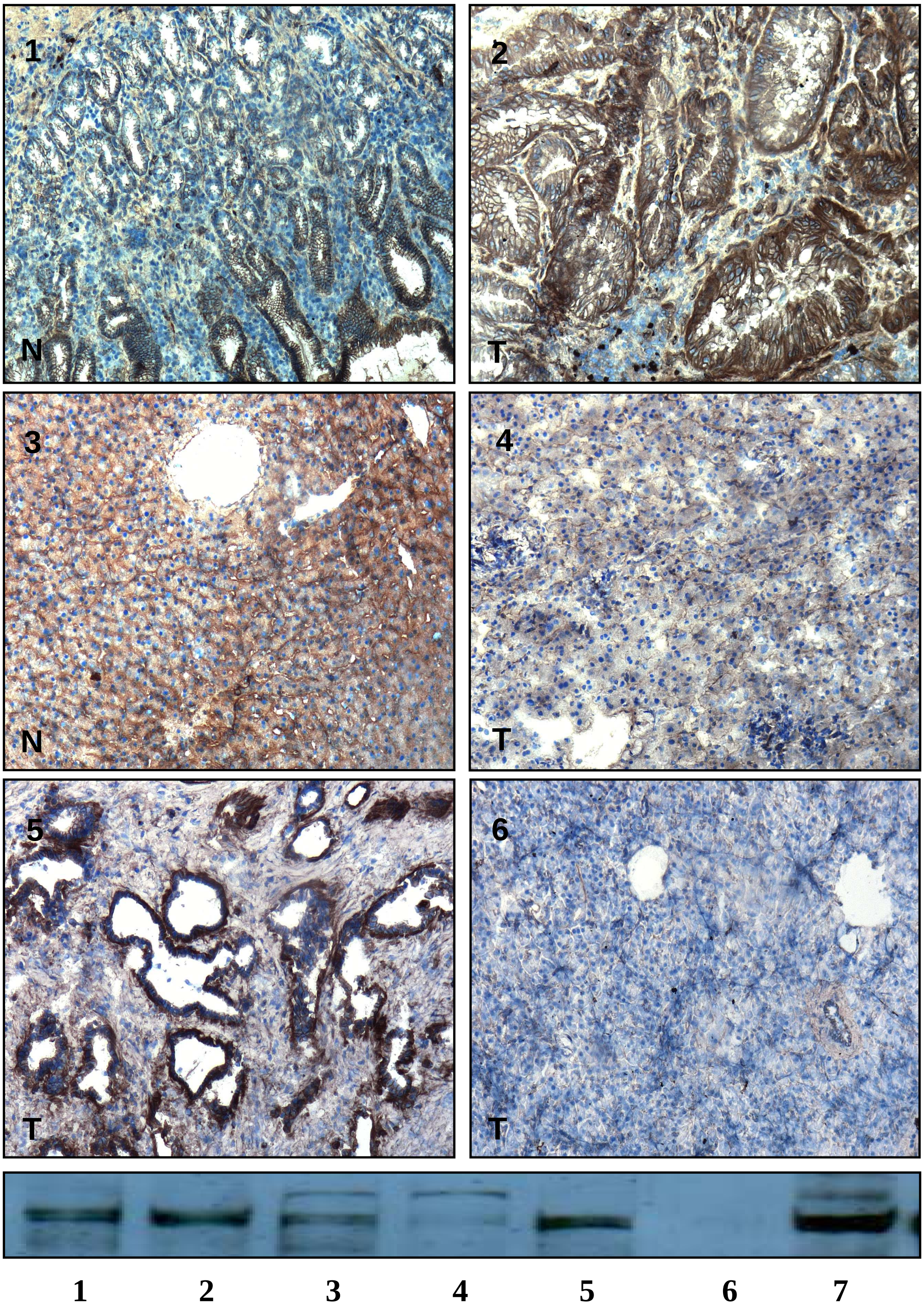


***Figure 2*: The consistent incidence of EphA2 in immunohistochemical staining and western blot.** For each type of tissue, same two sections were performed by IHC and Western Blot, respectively. 1 = normal corpus; 2 = gastric adenocarcinoma; 3 = normal liver; 4 = hepatocellular cell carcinoma; 5 = cholangiocarcinoma; 6 = normal pancreas; 7 = pancreatic adenocarcinoma. * N = normal; T = carcinoma * Due to tissue lack, no corresponding pancreatic adenocarcinoma section was available for IHC detection.

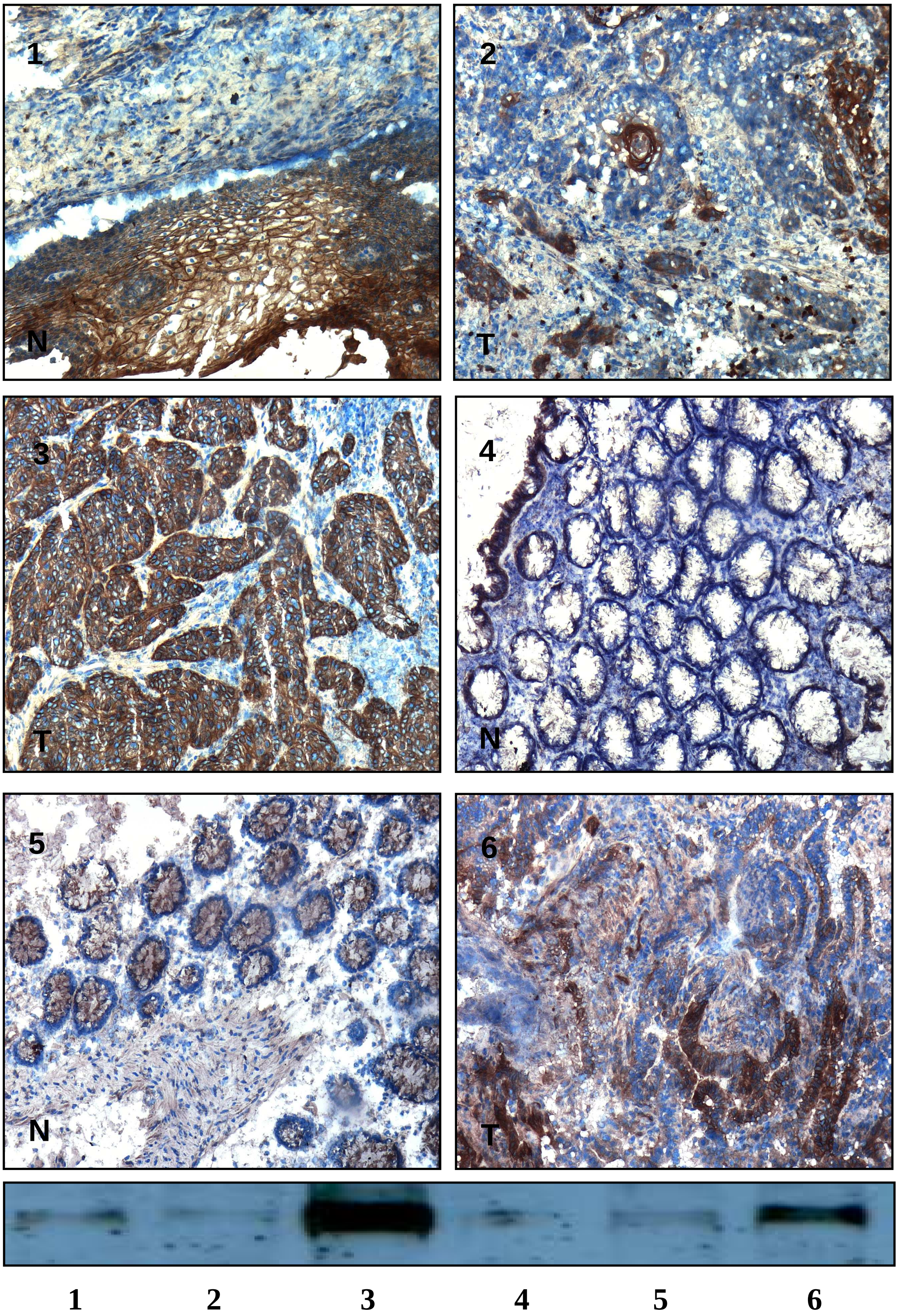


***Figure 3*: The consistent incidence of EphA2 in immunohistochemical staining and western blot.** For each type of tissue, same two sections were performed by IHC and Western Blot, respectively. 1 = normal esophagus; 2 = esophageal squamous cell carcinoma; 3 = esophageal adenocarcinoma; 4 = normal rectum; 5 = normal colon; 6 = colorectal adenocarcinoma * N = normal; T = carcinoma

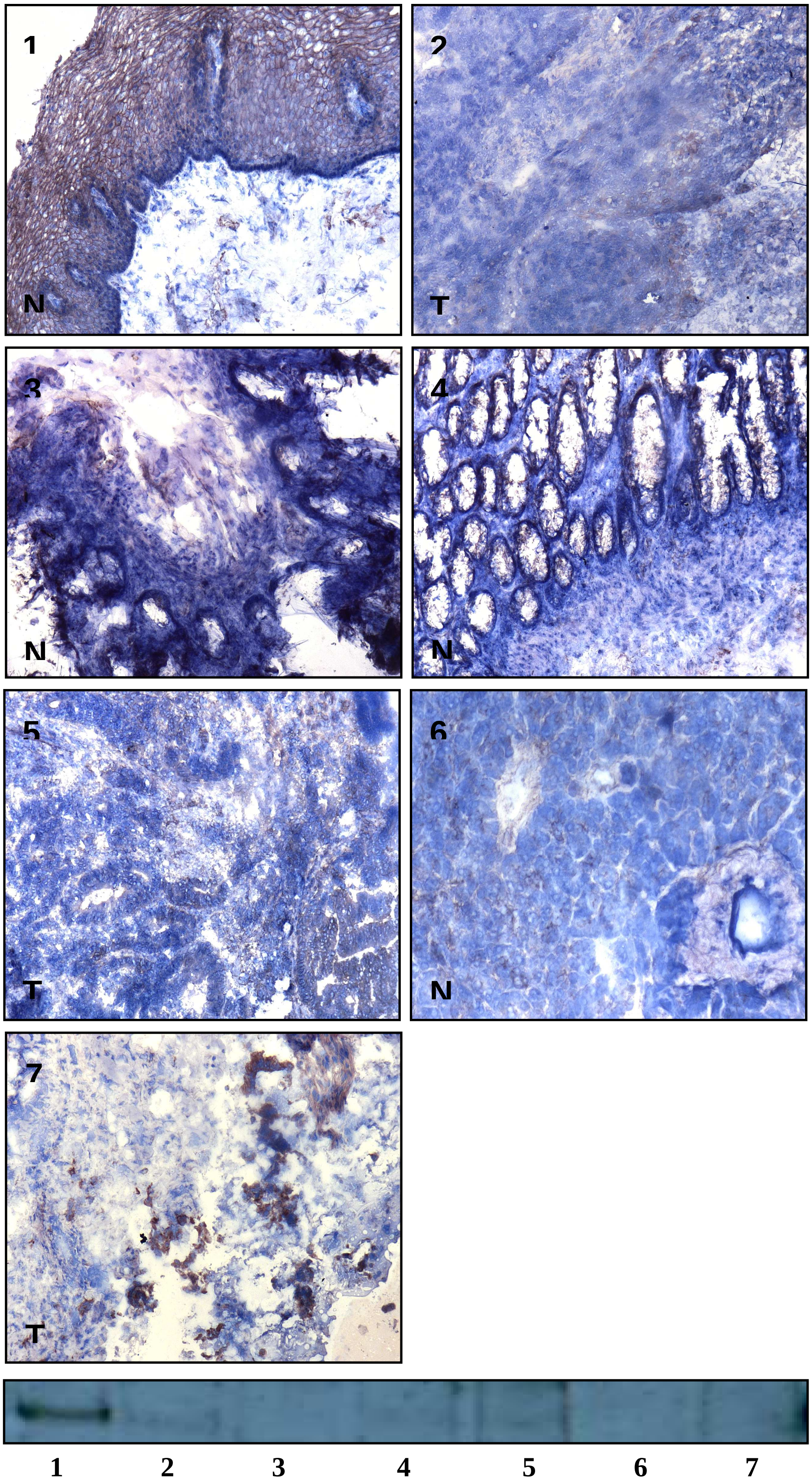


***Figure 4*: The consistent incidence of EphA2 in immunohistochemical staining and western blot.** For each type of tissue, same two sections were performed by IHC and Western Blot, respectively. 1 = normal esophagus; 2 = esophageal squamous cell carcinoma; 3 = normal colon; 4 = normal rectum; 5 = colorectal carcinoma; 6 = normal pancreas;7 = pancreatic adenocarcinoma * N = normal; T = carcinoma

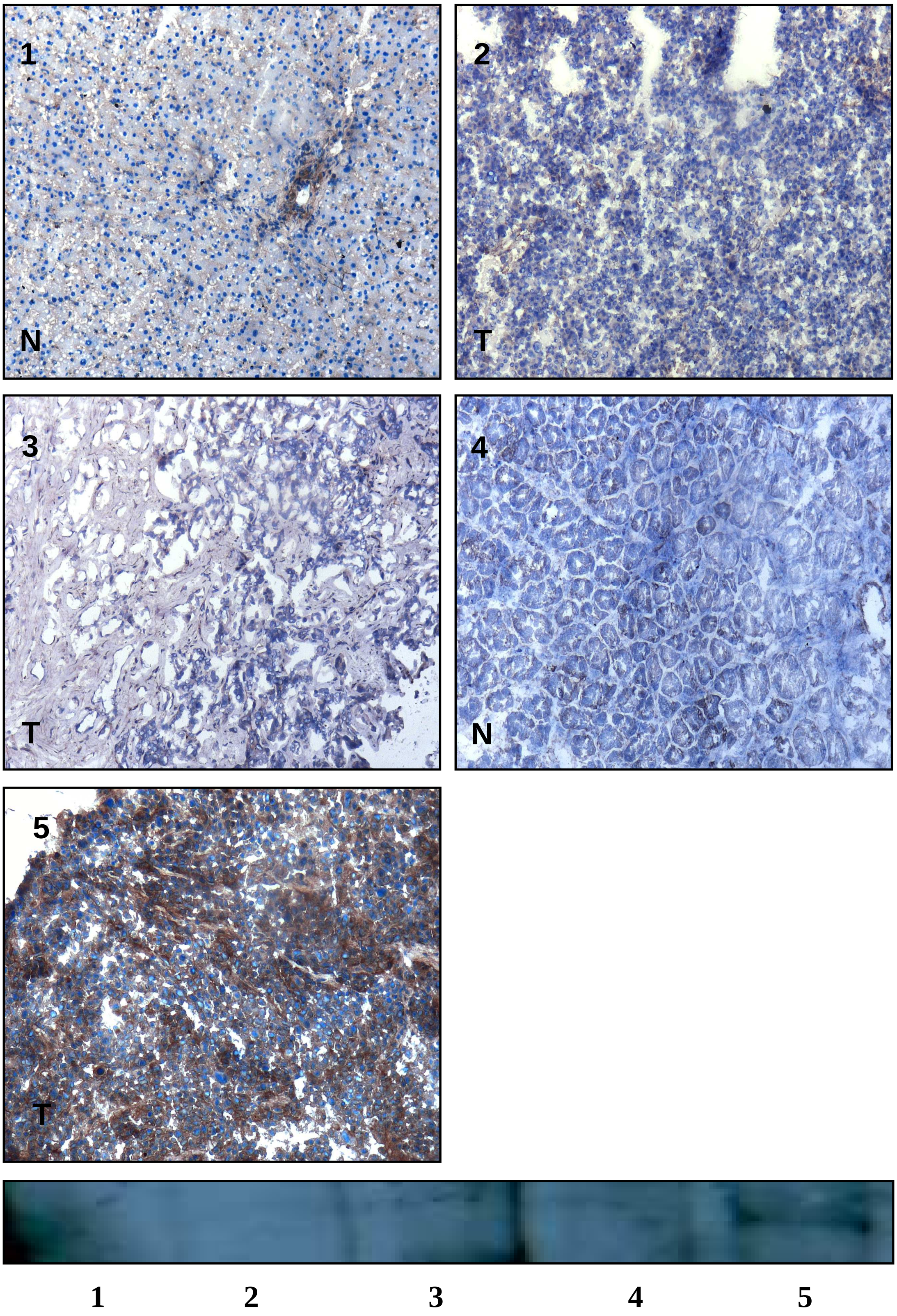


***Figure 5*: The consistent incidence of EphA2 in immunohistochemical staining and western blot.** For each type of tissue, same two sections were performed by IHC and Western Blot, respectively. 1 = normal liver; 2 = hepatocellular cell carcinoma; 3 = cholangiocarcinoma; 4 = normal stomach; 5 = stomach adenocarcinoma * N = normal; T = carcinoma

4.2 The expression pattern of EphA2 receptor was investigated in micro arrayed digestive system organs and corresponding carcinomas by immunohistochemical staining

The first and crucial step before the ultimate clinical therapeutic and diagnostic utilization of a target was for us to get an overview of how the target of interest is contrasted in normal and diseased cells and how this target is engaged in the development of tumor. To this end, we carried out the immunohistochemical staining by using micro arrayed pathological models to evaluate the expression pattern of EphA2 in normal versus carcinoma tissues in eight kinds of carcinomas, and in every type of carcinoma being classified into well, moderate and poor differentiations.

4.2.1 Cellular localization of EphA2 receptor

The following micro arrayed normal and carcinoma tissues from seven organs were examined by immunohistochemical staining. The localization of EphA2 was significantly different between normal and malignant epitheliums in cardia and pancreas. In normal cardia EphA2 was found to mainly express on the membrane, whereas in the case of malignant cardia most EphA2 was settled at intracellular sites. Pancreatic adenocarcinoma expressed EphA2 more often on the membrane, yet normal pancreas fixed EphA2 primarily on the inside of the cells. No significance of localization was discovered in other normal and corresponding carcinoma tissues, where EphA2 was defined either on plasma membrane or at intracellular sites, or at both sites *(Table 3).*

Tissue type	N	Negative	Positive			*p* value
			membrane	intracellular	both	
Normal esophagus	34	0	9	0	25	*0.098*
Esophageal squamous cell carcinoma	30	0	3	2	25	
Normal esophagus	7	0	7	0	0	*0.091*
Esophageal adenocarcinoma	9	1	4	3	1	
Normal cardia	5	2	3	0	0	***0.038***
Cardia adenocarcinoma	22	6	3	12	1	
Normal stomach	8	5	2	1	0	*0.327*
Stomach adenocarcinoma	24	8	4	10	2	
Normal colon	3	2	0	1	0	*0.243*
Colorectal adenocarcinoma	45	33	7	1	4	
Normal pancreas	26	6	5	15	0	***0.005***
Pancreatic adenocarcinoma	27	5	14	5	3	
Normal bile duct	3	1	0	2	0	*0.143*
Cholangiocarcinoma	46	4	11	16	15	
Normal liver	2	1	*0*	1	0	*1.000*
Hepatocellular carcinoma	49	19	*7*	21	2	

Table 3**: Comparison of three models of cellular localization of EphA2 between normal and**

carcinoma tissues. EphA2 was detectable on plasma membrane, in the intracellular compartment, or at both sites of normal and malignant cells. Significant differences were observed between normal cardia and adenocarcinoma of the cardia, and normal pancreas and pancreatic adenocarcinoma.

4.2.2 Expression patterns of EphA2 receptor

4.2.2.1 Comparison of expression rates in normal versus carcinoma tissues

In terms of the membrane expression rate among eight kinds of paired tissues, none showed significant difference between normal and carcinoma other than pancreas. EphA2 was found to express more frequently in pancreatic adenocarcinoma than in normal pancreas, by a percentage of 62.96 % versus 19.23 %. However, when it comes to the intracellular expression rate, cardia took over the character of pancreas by cardia adenocarcinoma expressing higher incidence of EphA2 than normal cardia, with a percentage of 59.09 % versus 0 % *(Table 4).*

Tissue type		**Membrane expression rates**		***p* value**	**Intracellular expression rates**		***p* value**
	N	**-**	**+**		**-**	**+**	
Normal esophagus	34	0 (0%)	34 (100%)	*0.216*	9 (26.5%)	25 (73.5%)	*0.173*
Esophageal squamous cell carcinoma	30	2 (6.7%)	28 (93.3%)		3 (10%)	27 (90%)	
Normal esophagus	7	0 (0%)	7 (100%)	*0.088*	7 (100%)	0 (0%)	*0.088*
Esophageal adenocarcinoma	9	4 (44.4%)	5 (55.6%)		5 (55.6%)	4 (44.4%)	
Normal cardia	5	2 (40%)	3 (60%)	*0.091*	5 (100%)	0 (0%)	***0.041***
Cardia adenocarcinoma	22	18 (81.8%)	4 (18.2%)		9 (40.9%)	13 (59.1%)	
Normal stomach	8	6 (75%)	2 (25%)	*1.000*	7 (87.5%)	1 (12.5%)	*0.101*
Stomach adenocarcinoma	24	18 (75%)	6 (25%)		12 (50%)	12 (50%)	
Normal colon	3	3 (100%)	0 (0%)	*1.000*	2 (66.7%)	1 (33.3%)	*0.336*
Colorectal adenocarcinoma	45	34 (75.6%)	11 (24.4%)		40 (88.9%)	5 (11.1%)	
Normal pancreas	26	21 (80.8%)	5 (19.2%)	***0.003***	11 (42.3%)	15 (57.7%)	*0.075*
Pancreatic adenocarcinoma	27	10 (37%)	17 (63%)		19 (70.4%)	8 (29.6%)	
Normal bile duct	3	3 (100%)	0 (0%)	*0.110*	1 (33.3%)	2 (66.7%)	*1.000*
Cholangiocarcinoma	46	21 (45.7%)	25 (54.3%)		15 (32.6%)	31 (67.4%)	
Normal liver	2	2 (100%)	0 (0%)	*1.000*	1 (50%)	1 (50%)	*1.000*
Hepatocellular carcinoma	49	40 (81.6%)	9 (18.4%)		26 (53.1%)	23 (46.9%)	

***Table 4*: Comparison of the expression rate of EphA2 in normal versus carcinoma tissues.** The cellular localization of EphA2 was classified into plasma membrane and intracellular part. The incidence of EphA2 in normal and corresponding carcinoma tissues was roughly divided into negative and positive, regardless of the actual diverse positive expression degree. Significant differences existed between normal cardia and cardia adenocarcinoma, and normal pancreas and pancreatic adenocarcinoma.

4.2.2.2 Comparison of expression levels in normal versus carcinoma tissues

Compared to the simple pattern of expression rates, the expression level showed much complexity. Normal esophagus expressed higher EphA2 on plasma membrane than esophageal squamous cell carcinoma, whereas no difference was found between these two candidates for intracellular EphA2. Unlike squamous cell carcinoma, esophageal adenocarcinoma displays the expression pattern rather depending on the EphA2 localization. On the one hand on plasma membrane, it expressed EphA2 less than normal esophagus, on the other hand at intracellular part, it expressed more EphA2 than normal esophagus. For cardia, concerning the EphA2 level on plasma membrane, there was no difference between normal and malignancy, whereas substantial difference was found with respect to the intracellular part, with cardia adenocarcinoma expressing more EphA2 than normal cardia. Considering plasma membrane expression, significance was displayed between normal and malignancy, with a pattern of pancreatic adenocarcinoma expressing more EphA2 than normal pancreas. In contrast, in the intracellular part, normal pancreas expressed higher EphA2 than pancreatic adenocarcinoma *(Table 5)*.

Tissue type	**Membrane expression level**					***p* value**	**Intracellular expression level**				***p* value**
	N	-	+	++	+++		-	+	++	+++	
Nomral esophagus	34	0	0	6	28	***0.001***	9	1	6	18	*0.611*
Esophageal squamous cell carcinoma	30	2	2	13	13		3	0	12	15	
Normal esophagus	7	0	0	5	2	***0.011***	7	0	0	0	***0.049***
Esophageal adenocarcinoma	9	4	1	4	0		5	0	4	0	
Normal cardia	5	2	1	1	1	*0.062*	5	0	0	0	***0.025***
Cardia adenocarcinoma	22	18	1	2	1		9	4	9	0	
Normal stomach	8	6	1	1	0	*0.863*	7	0	1	0	*0.069*
Stomach adenocarcinoma	24	18	1	3	2		12	1	9	2	
Normal colon	3	3	0	0	0	*0.338*	2	1	0	0	*0.353*
Colorectal adenocarcinoma	45	34	0	8	3		40	0	5	0	
Normal pancreas	26	21	1	0	4	***0.005***	11	0	14	1	***0.038***
Pancreatic adenocarcinoma	27	10	1	10	6		19	1	6	1	
Normal bile duct	3	3	0	0	0	*0.089*	1	0	2	0	*0.834*
Cholangoicarcinoma	46	21	2	16	7		15	1	25	5	
Normal liver	2	2	0	0	0	*0.510*	1	0	1	0	*1.000*
Hepatocellular carcinoma	49	40	0	7	2		26	3	14	6	

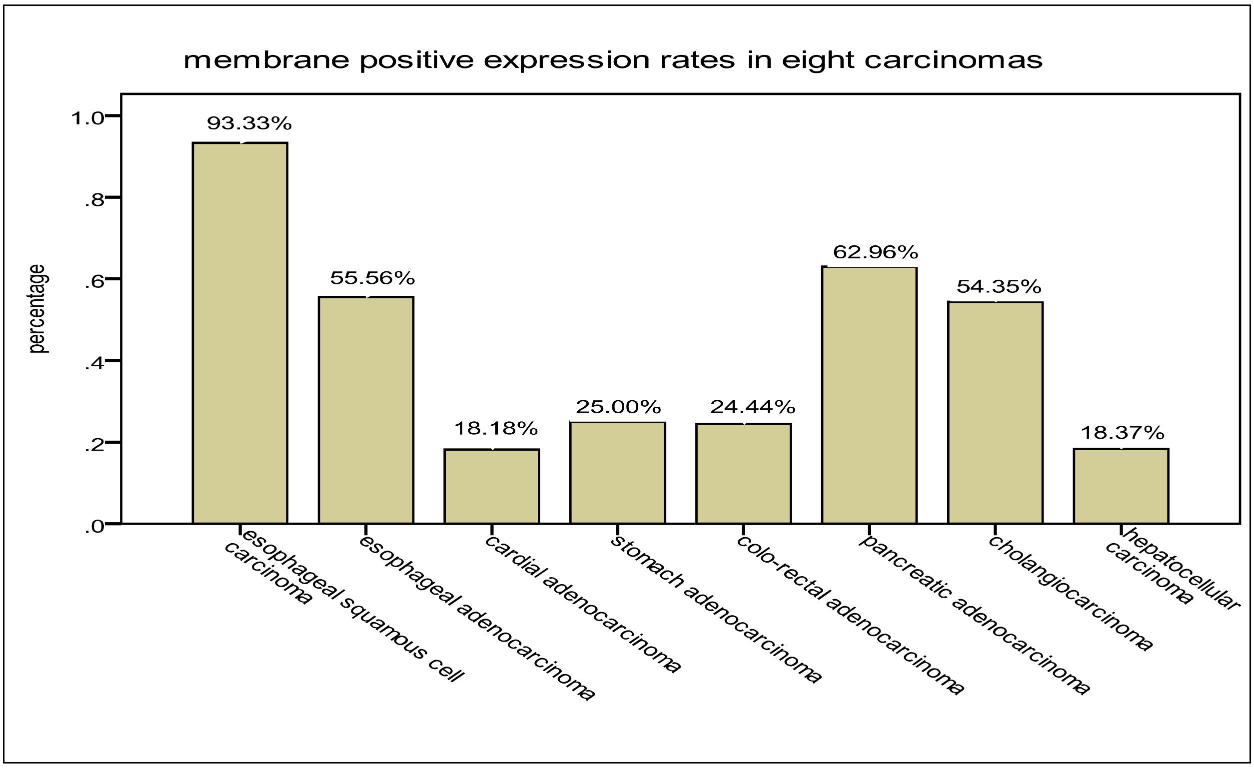
Table 5**: Comparison of the expression level of EphA2 between normal and carcinoma tissues.** The cellular localization of EphA2 was classified into plasma membrane and intracellular part. Significant differences were found in normal esophagus versus esophageal squamous cell carcinoma, normal esophagus versus esophageal adenocarcinoma, normal cardia versus cardia adenocarcinoma, and in normal pancreas versus pancreatic adenocarcinoma.

4.2.2.3 Comparison of expression rates in eight carcinoma tissues

Briefly, the eight carcinoma entities can be divided into three pathological kinds: squamous cell carcinoma, adenocarcinoma, and hepatocellular carcinoma. To investigate whether EphA2 was distinctively expressed among these eight carcinomas, we arrayed the eight candidates together and made a comparison. First of all, we compared the plasma membrane expression rates of EphA2 in these eight carcinomas. Generally, we found that squamous cell carcinoma was the tissue type which expressed EphA2 at the highest frequency, adenocarcinoma ranked second, and hepatocellular carcinoma expressed the least. In detail, the incidence of EphA2 was remarkably higher in esophageal squamous cell carcinoma than in four adenocarcinomas, including cardia adenocarcinoma, stomach adenocarcinoma, colorectal adenocarcinoma and cholangiocarcinoma. However, esophageal squamous cell carcinoma was not able to compete with pancreatic adenocarcinoma because no statistical difference was observed between these two contestants. In addition, for esophagus itself, no contrast was found between squamous cell carcinoma and adenocarcinoma. Three kinds of carcinomas: esophageal squamous cell carcinoma, pancreatic adenocarcinoma, and cholangiocarcinoma, presented EphA2 at a markedly higher frequency than hepatocellular carcinoma. Secondly, we compared the intracellular expression rates of these eight candidates. Among different carcinoma type, esophagus expressed more EphA2 than colon-retum, pancreas and liver. As to the competition within the adenocarcinoma, cardia as well as stomach and bile duct had a higher ratio of EphA2 than colon-retum. Hepatocellular carcinoma displayed a much higher EphA2 incidence than colorectal adenocarcinoma *(Table 6, Fig.6).*

Grey part = membrane ***White part = intracellular***	***N***	***p* value (α =0.001724)**							
		ESCC	EAC	CAC	SAC	CRAC	PAC	CCC	HCC
Esophageal squamous cell carcinoma (ESCC)	30		*0.018*	***0.000***	***0.000***	***0.001***	*0.013*	***0.000***	***0.000***
Esophageal adenocarcinoma (EAC)	9	*0.009*		*0.077*	*0.121*	*0.106*	*0.712*	*1.000*	*0.030*
Cardia adenocarcinoma (CAC)	22	*0.023*	*0.693*		*0.725*	*0.757*	*0.004*	*0.010*	*1.000*
Stomach adenocarcinoma (SAC)	24	*0.003*	*1.000*	*0.747*		*1.000*	*0.015*	*0.036*	*0.547*
Colorectal adenocarcinoma (CRAC)	45	***0.000***	*0.033*	***0.000***	***0.001***		*0.003*	*0.007*	*0.641*
Pancreatic adenocarcinoma (PAC)	27	***0.000***	*0.443*	*0.075*	*0.230*	*0.062*		*0.636*	***0.000***
Cholangiocarcinoma (CCC)	46	*0.047*	*0.261*	*0.690*	*0.246*	***0.000***	*0.004*		***0.001***
Hepatocellular carcinoma (HCC)	49	***0.000***	*1.000*	*0.490*	*1.000*	***0.000***	*0.220*	*0.071*	

***Table 6*: Comparison of the expression rate of EphA2 in each paired carcinomas.** The table was divided into two parts with reference to EphA2 cellular localization — plasma membrane and intracellular part. Firstly, the statistical significance of the expression rate of EphA2 among eight types of carcinoma was evaluated by Chi-square test and Fisher's exact test. Secondly, partitions of Chi-square method were used for the comparison in each single pair of carcinoma. The tablet just shows the results from Partitions of Chi-square method.



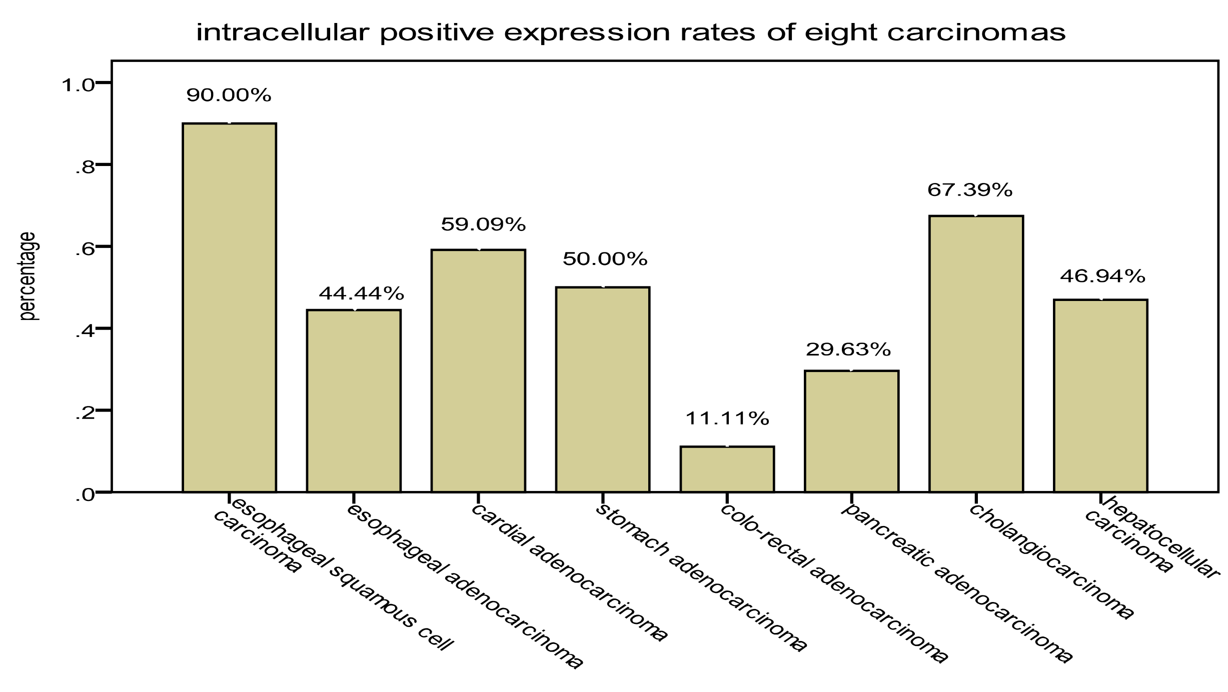


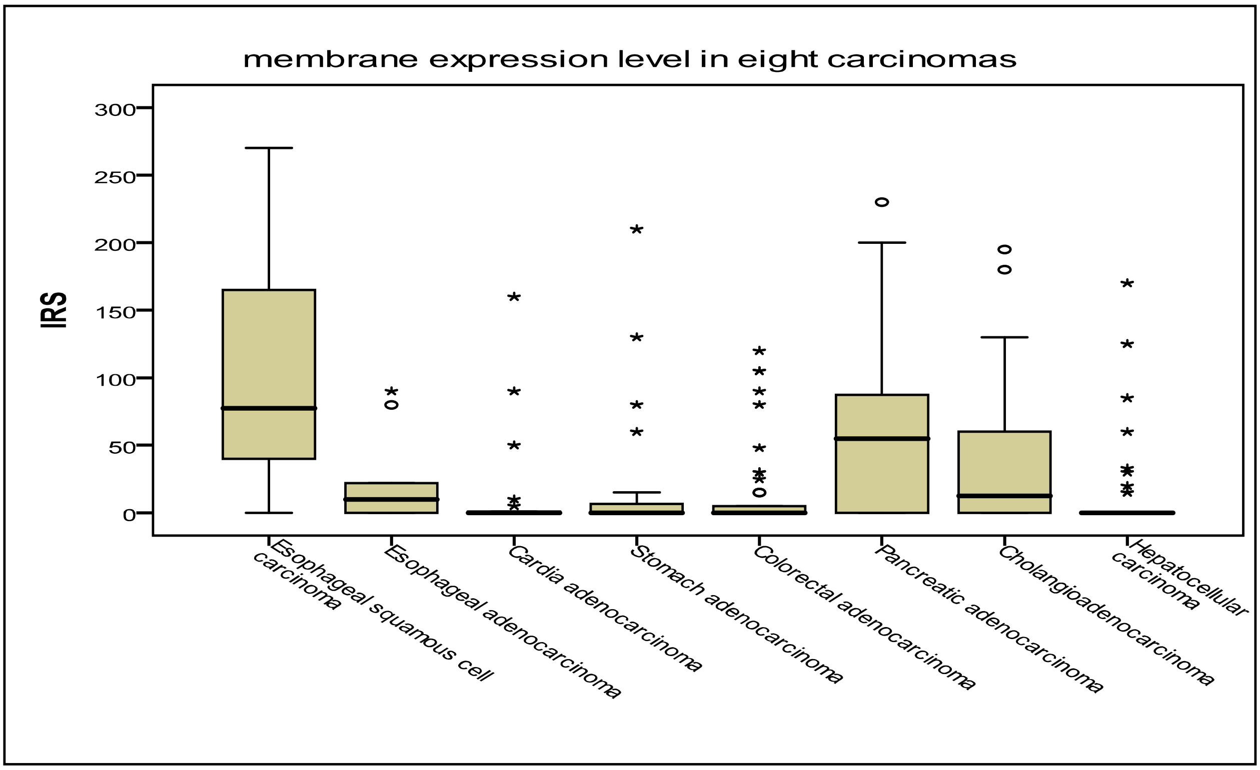
***Figure 6*: Membrane and intracellular expression rates of EphA2 in eight carcinomas.** Based on the EphA2 cellular localization — plasma membrane and intracellular part, the graph was separately illustrated.

4.2.2.4 Comparison of expression levels in eight carcinoma tissues

As usual, we detected the distinctive profile of expression level of EphA2 among these eight carcinoma candidates. Like expression rates we mentioned above, squamous cell carcinoma still represented the strongest entity in terms of the expression degree, irrespective of the localization of EphA2. On membrane, esophageal squamous cell carcinoma displayed a much higher level of EphA2 than cardia, stomach, colorectal adenocarcinoma and hepatocellular carcinoma. At the intracellular compartment, apart from expressing more EphA2 than cardia, stomach, colo-retum and liver malignant cells, esophageal squamous cell carcinoma also demonstrated a higher degree of EphA2 than pancreatic adenocarcinoma. Cholangiocarcinoma expressed more EphA2 than colorectal adenocarcinoma *(Table 7, Fig. 7)*.

Grey part = membrane ***White part = intracellular***	***p* value (α =0.05)**								
	N	ESCC	EAC	CAC	SAC	CRAC	PAC	CCC	HCC
Esophageal squamous cell carcinoma (ESCC)	30		*0.42*	***0.00***	***0.00***	***0.00***	*0.49*	0.05	***0.00***
Esophageal adenocarcinoma (EAC)	9	*0.17*		*0.95*	*0.99*	*0.99*	*1.00*	*1.00*	*0.94*
Cardia adenocarcinoma (CAC)	22	***0.03***	*1.00*		*1.00*	*1.00*	*0.17*	*0.37*	*1.00*
Stomach adenocarcinoma (SAC)	24	***0.03***	*1.00*	*1.00*		*1.00*	*0.37*	*0.66*	*1.00*
Colorectal adenocarcinoma (CRAC)	45	***0.00***	*0.92*	*0.37*	*0.31*		*0.19*	*0.40*	*1.00*
Pancreatic adenocarcinoma (PAC)	27	***0.00***	*1.00*	*0.97*	*0.96*	*0.97*		*1.00*	0.05
Cholangoicarcinoma (CCC)	46	*0.18*	*0.97*	*0.97*	*0.97*	***0.00***	*0.24*		*0.13*
Hepatocellular carcinoma (HCC)	49	***0.00***	*1.00*	*1.00*	*1.00*	*0.13*	*0.94*	*0.85*	

***Table 7*: Comparison of the expression level of EphA2 in each paired carcinomas.** The table was divided into two parts with reference to EphA2 cellular localization— plasma membrane and intracellular part. Firstly, the statistical significance of IRS values among eight types of carcinoma was evaluated by Kruskal-Wallis H test. Then, Nemenyi test was used for the comparison in each single pair of carcinoma. The tablet just shows the results from Nemenyi test.



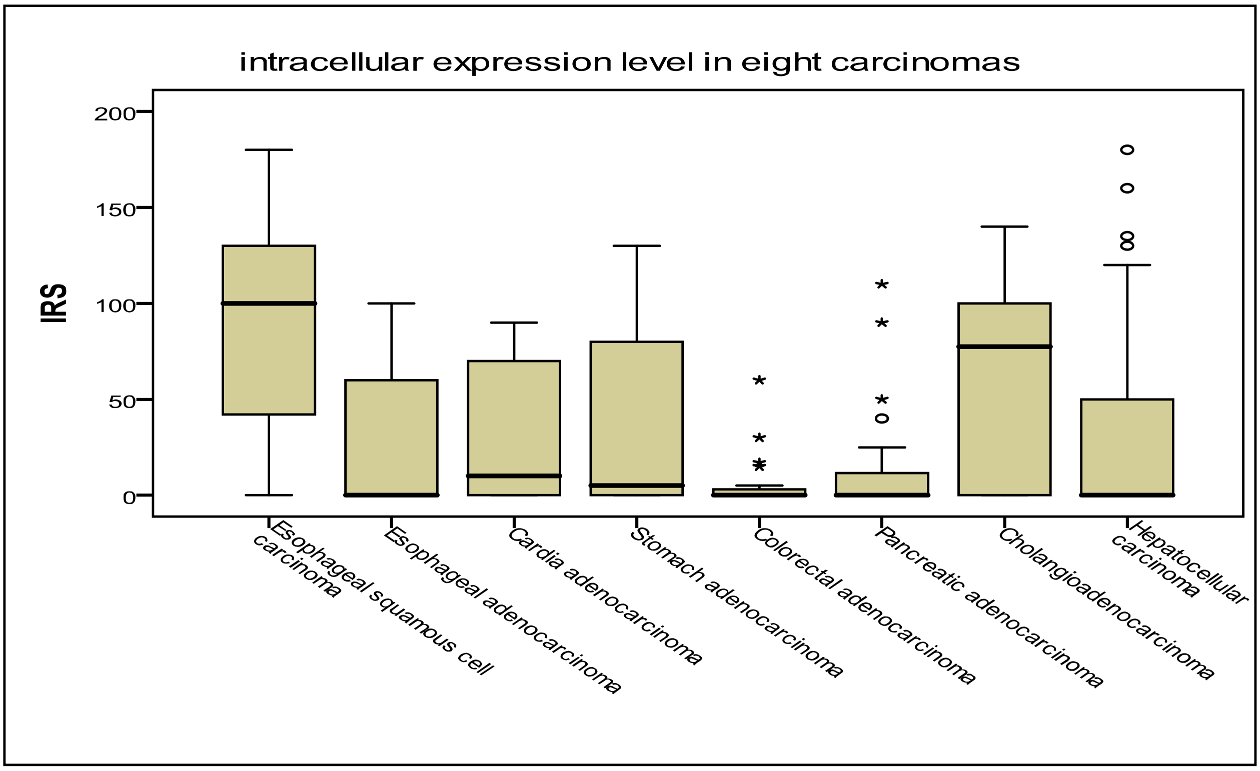


***Figure 7*: Membrane and intracellular expression levels of EphA2 in eight carcinoma tissues.** The graph was separately illustrated with reference to EphA2 cellular localization — plasma membrane and intracellular part. The expression level of EphA2 is reflected by the IRS values. Yellow bar stands for IRS values between no. 25 and no. 75 percentile. Outliers are displayed by star and medians are shown by bold slash.

4.2.2.5 Comparison of expression rates and levels among three histological differentiations in eight carcinoma tissues

Eight carcinoma tissues were individually divided into three groups according to the differentiation degree: well differentiation, moderate differentiation and poor differentiation. After making a statistical analysis, it was found that among the three differentiations of each kind of carcinoma, neither the location of EphA2 nor the expression rate and level was shown to have any statistically meaningful difference *(Table 8, Table 9).*

Tissue type		Membrane expression rates		p value	Membrane expression intensity				p value
	N	-	+		-	+	++	+++	
Esophageal squamous cell carcinoma	30			*0.241*					*0.547*
Well	3	1 (33.3%)	2 (66.7%)		1	0	0	2	
Moderate	20	1 (5%)	19 (95%)		1	0	9	10	
Poor	7	0 (0%)	7 (100%)		0	2	3	2	
Esophageal adenocarcinoma	9			*1.000*					*0.565*
Well	1	1 (100%)	0 (0%)		1	0	0	0	
Moderate	1	0 (0%)	1 (100%)		0	1	0	0	
Poor	7	3 (42.9%)	4 (57.1%)		3	0	4	0	
Cardial adenocarcinoma	22			*0.478*					*0.389*
Well	8	7 (87.5%)	1 (12.5%)		7	0	1	0	
Moderate	4	4 (100%)	0 (0%)		4	0	0	0	
Poor	10	7 (70%)	3 (30%)		7	1	1	1	
Stomach adenocarcinoma	24			*0.797*					*0.449*
Well	3	3 (100%)	0 (0%)		3	0	0	0	
Moderate	5	4 (80%)	1 (20%)		4	1	0	0	
Poor	16	11 (68.8%)	5 (31.3%)		11	0	3	2	
Colo-rectal adenocarcinoma	45			*0.683*					*0.523*
Well	6	4 (66.7%)	2 (33.3%)		4	0	1	1	
Moderate	36	27 (75%)	9 (25%)		27	0	7	2	
Poor	3	3 (100%)	0 (0%)		3	0	0	0	
Pancreatic adenocarcinoma	27			*0.210*					*0.387*
Well	1	1 (100%)	0 (0%)		1	0	0	0	
Moderate	17	7 (41.2%)	10 (58.8%)		7	1	5	4	
Poor	9	2 (22.2%)	7 (77.8%)		2	0	5	2	
Cholangoicarcinoma	46			*0.425*					*0.309*
Well	6	4 (66.7%)	2 (33.3%)		4	0	2	0	
Moderate	29	13 (44.8%)	16 (55.2%)		13	1	12	3	
poor	11	4 (36.4%)	7 (63.6%)		4	1	2	4	
Hepatocellular carcinoma	49			*0.305*					*0.270*
Well	5	5 (100%)	0 (0%)		5	0	0	0	
Moderate	17	12 (70.6%)	5 (29.4%)		12	0	4	1	
Poor	27	23 (85.2%)	4 (14.8%)		23	0	3	1	

***Table 8*: Comparison of the expression rate and level of plasma membrane localized EphA2 among three types of differentiation in eight carcinomas.** No significance of the correlation between expression rate of EphA2 and histological differentiation was found in these eight carcinomas, as well, the expression levels of EphA2 were similar among three histological differentiations for all carcinomas.

Tissue type		**Intracellular expression rates**		***p* value**	**Intracellular expression level**				***p* value**
	N	**-**	**+**		**-**	**+**	**++**	**+++**	
Esophageal squamous cell carcinoma	30 3			*1.000*					*0.422*
Well	20	0 (0%)	3 (100%)		0	0	1	2	
Moderate	7	2 (10%)	18 (90%)		2	0	7	11	
Poor		1 (14.3%)	6 (85.7%)		1	0	4	2	
Esophageal adenocarcinoma	9			*0.167*					*0.240*
Well	1	0 (0%)	1 (100%)		0	0	1	0	
Moderate	1	0 (0%)	1 (100%)		0	0	1	0	
Poor	7	5 (71.4%)	2 (28.6%)		5	0	2	0	
Cardial adenocarcinoma	22			*0.146*					*0.636*
Well	8	3 (37.5%)	5 (62.5%)		3	1	4	0	
Moderate	4	0 (0%)	4 (100%)		0	3	1	0	
Poor	10	6 (60%)	4 (40%)		6	0	4	0	
Stomach adenocarcinoma	24			*0.714*					*0.651*
Well	3	2 (66.7%)	1 (33.3%)		2	0	1	0	
Moderate	5	3 (60%)	2 (40%)		3	0	2	0	
Poor	16	7 (43.8%)	9 (56.3%)		7	1	6	2	
Colo-rectal adenocarcinoma	45			*0.258*					*0.382*
Well	6	5 (83.3%)	1 (16.7%)		5	0	1	0	
Moderate	36	33 (91.7%)	3 (8.3%)		33	0	3	0	
Poor	3	2 (66.7%)	1 (33.3%)		2	0	1	0	
Pancreatic adenocarcinoma	27			*0.240*					*0.245*
Well	1	1 (100%)	0 (0%)		1	0	0	0	
Moderate	17	10 (58.8%)	7 (41.2%)		10	1	5	1	
Poor	9	8 (88.9%)	1 (11.1%)		8	0	1	0	
Cholangoicarcinoma	46			*1.000*					*0.907*
Well	6	2 (33.3%)	4 (66.7%)		2	0	4	0	
Moderate	29	10 (31%)	19 (69%)		10	1	14	4	
Poor	11	3 (27.3%)	8 (72.7%)		3	0	7	1	
Hepatocellular carcinoma	49			*0.916*					*0.866*
Well	5	3 (60%)	2 (40%)		3	0	1	1	
Moderate	17	18 (47.1%)	9 (52.9%)		18	1	6	2	
Poor	27	15 (55.6%)	12 (44.4%)		15	2	7	3	

***Table 9*: Comparison of the expression rate and level of intracellular EphA2 among three types of differentiation in eight carcinomas.** No significance of the correlation between expression rate of EphA2 and histological differentiation was found in these eight carcinomas, and the expression levels of EphA2 were similar among three histological differentiations for all carcinomas.

5. Discussion

5.1 The major findings in this work

The major findings in this work are that: 1) EphA2 can be present either on plasma membrane or in the intracellular part of normal and malignant epithelial cells, or both. 2) EphA2 was found to be overexpressed in esophageal, cardia and pancreatic adenocarcinoma in contrast to their corresponding adjacent normal tissues. Furthermore, the localization of EphA2 differed in these three carcinomas. It was overexpressed at the intracellular part of malignant cells in esophageal and cardia adenocarcinoma, but on the plasma membrane of malignant cells in pancreatic adenocarcinoma. 3) EphA2 was expressed in a heterogeneous manner in various carcinomas. Esophageal squamous cell carcinoma was found to express higher EphA2 than five types of carcinomas, including cardia, stomach, colorectal, pancreatic adenocarcinoma and hepatocellular carcinoma, though it expressed lower EphA2 than normal esophagus. More expression of EphA2 was displayed in cholangiocarcinoma than in colorectal adenocarcinoma. 4) No correlation between the EphA2 expression degree and the histological differentiation was found in any of the different kinds of carcinomas.

5.2 Regulation of EphA2 expression

As a transmembrane tyrosine kinase receptor, EphA2 critically controls many aspects of cell behavior. In normal cells, EphA2 expression appears to be restricted to the intercellular junctions between epithelial cells, where it binds ligands that are anchored to the membrane of adjacent cells. Ligand binding causes EphA2 to become phosphorylated and then internalized and degraded, which results in the maintenance of a low level of EphA2 expression on the surface of cells. In malignant cells, due to the loss of E-cadherin expression or function, the normal epithelial cell-cell contacts are often disrupted, which destabilizes EphA2 ligand binding and thus EphA2 is able to redistribute across the cell surface and positively regulate malignant cell growth and invasion in a non-tyrosine phosphorylated way [41].

The exact mechanism that governs EphA2 overexpression has yet to be fully elucidated. It has been confirmed that EphA2 is a direct transcriptional target of the Ras-MAPK pathway, and that is overexpressed in Ras-transformed cells and in Ras overexpressing transgenic mice [87, 88]. Thus, EphA2 is transcriptionally up-regulated during the process of malignant transformation and progression, potentially as a result of aberrant growth factor signaling originating through another RTK. A number of studies have shown that epithelial growth factor receptor (EGFR) activation regulates EphA2 expression in cancer [89]. It is also shown that EphA2 is a target gene of the p53 family, and that an increase of EphA2 transcript levels correlates with an increase of EphA2 protein expression in response to DNA damage corresponds with p53 activation, providing another possible mechanism for EphA2 overexpression [90]. In addition, EphA2 is tightly regulated by homeobox transcription factors, some of which are up-regulated during malignancy, and EphA2 could potentially be a target gene during this process [91]. Other possible contributing factors to the abundance of EphA2 in malignancies are gene mutation and amplification, which have not been confirmed by enough evidence [92].

In this study it was found that besides overexpression in several carcinomas, high levels of EphA2 were also found on the plasma membrane in normal esophageal squamous epithelium and in intracellular compartments in normal pancreatic ductal epithelium, where higher EphA2 levels were observed than in their relative carcinomas. No expression difference was found between the normal and corresponding malignant epitheliums of stomach, colon-rectum, bile duct and liver. These demonstrations seem contradictory to the scenario that EphA2 is commonly absent or lower expressed in normal cells, compared to malignancies. Our point of view is that several aspects may explain this phenomenon. First, the normal tissues recruited in our study are adjacent normal tissues which means they come from the very near surrounding region of carcinoma lesions. Genetically, such kind of adjacent normal tissues are not real representatives of normal tissues and probably have already undergone a series of chromosomal alterations within the cells in some degree and therefore been directed to a state of primary malignant transformation. In addition to malignant transformation, gene mutation and amplification contribute to the overexpression of EphA2. Second, though it is observed that EphA2 is massively downregulated in normal adult tissue but up-regulated in malignancy, decreased Eph receptor expression in malignant cancer cell lines and tumor specimens has also been reported. For example, EphA1 is downregulated in advanced human skin and colorectal cancers [93, 94]. EphB6 expression is lower in metastatic than non-metastatic lung cancers [95]. Recent studies show that an initial Eph receptor upregulation, which is due to activated oncogenic signaling pathways and other factors, can be followed by epigenetic silencing in more advanced stages owing to promoter hypermethylation [94, 96-98]. Transcriptional repression, such as repression of EphB2 by REL (a member of the nuclear factor-κB family) in colorectal cancer, may play a part in Eph silencing [99]. The findings focusing on other members of Eph receptor family remind us that it is not surprising that we found the EphA2 expression level decreased in our carcinoma specimens as well. Third, in normal adult tissues, expression of EphA2 is regarded to be limited in those tissues having high proportion of dividing epithelial cells, such as skin, lung and intestine [100]. The object of our research is restricted to digestive system organs where many kinds of constantly dividing epithelium are lining. Furthermore, there is one study that showed that EphA2 was strongly expressed in normal colon epithelium and played a significant role in controlling colon crypt maturation, which is partly consistent with our results [61].

5.3 Three steps for identifying EphA2 for targeting strategy

5.3.1 Organ targeting

An active targeting may include three levels of action: first, organ targeting; second, cell targeting; third, intracellular targeting. As the first level, an organ screening aims to define organ types that are potentially particularly suitable to be targeted based on a specific targeting molecule. In this work it was found that only limited organs are accessible for a further EphA2 targeting project. It seems that most digestive system tissues lack a favorable contrast between normal and tumor tissue. As shown in table 5, no contrast of the EphA2 expression between normal and carcinoma tissues was identified in stomach, colon-rectum, bile duct or liver. Although esophageal squamous cell carcinoma has the strongest expression of EphA2 compared to other carcinomas as shown in figure 7, esophagus is not suitable as an organ target either, because normal esophagus expressed even more EphA2 on plasma membrane than esophageal squamous cell carcinoma (see table 5). Overall, an expansion of the tissue type from digestive system tissues to other human tissues is obviously necessary.

5.3.2 Cell targeting

Initially, EphA2 was first discovered in adult epithelial cells. Later studies showed that not only did tumor epithelium express a high level of EphA2, but tumor endothelium also expresses EphA2. A survey of EphA2 expression pattern in tumor vasculature revealed that EphA2 receptor and EphrinA1 ligand were consistently expressed in tumor associated endothelium from a variety of tumors [73]. Accordingly, EphA2 null endothelial cells failed to undergo cell migration and vascular assembly both *in vitro* and *in vivo* [101]. Tumor growth, angiogenesis, and metastasis were significantly inhibited in tumors orthotopically grafted to EphA2 deficient recipient mice [77]. In addition, EphA2 was identified as an important mediator of vasculogenic mimicry in highly aggressive uveal and cutaneous melanoma cells. Downregulation of EphA2 expression, using anti-sense oligonucleotide technology inhibited vasculogenic mimicry by aggressive melanoma cells [102]. These data demonstrate that not only EphA2 is expressed in tumor endothelial cells, but also it plays a functional role in tumor neovascularization that constitutes the most important part of tumor microenvironment.

This study revealed that in digestive system malignancies, EphA2 seemed preferentially being presented in squamous epithelial cell among various epitheliums. This conclusion is based on the results that the malignant squamous epithelium in esophageal squamous cell carcinoma expressed highest level of EphA2 in contrast with the non-squamous malignant epitheliums in five other types of carcinomas (see figures 6, 7). To confirm this postulation, further detection of EphA2 expression in other malignancies and comparison between squamous cell malignancies and non-squamous cell malignancies in these tissues are necessary, such as comparison between lung squamous cell carcinoma, cervix squamous cell carcinoma and skin squamous cell carcinoma on the one hand, and non-squamous cell carcinomas on the other hand.

That EphA2 can be expressed both in tumor cells and in endothelial cells makes EphA2 an excellent target for forming a “one stone, two birds” tumor targeting strategy. That means both tumor and tumor microenvironment can be targeted at the same time using one specific target.

5.3.3 Intracellular targeting

One of the most obvious strategies in tumor intracellular targeting is to couple chemotherapeutic, or radioactive and genetic therapeutic agents to targeting vehicles (selective agents) such as antibodies and peptides. By conjugating these two agents, the conjugate then may be used either as a contrast agent for molecular imaging or in treatments as chemotherapy, radiotherapy, and gene therapy. Receptor-mediated endocytosis is one of the main access for the intracellular localization of antibodies or peptides to carry out intracellular targeting. After binding to receptor targets on the plasma membrane, targeting agents are enabled to translocate over plasma membrane and arrive in the inner parts of cells, where the conjugates are released to perform their desired functions. In this regard, the plasma membrane location of targets is of great importance. To date, most EphA2 targeting strategies based their actions on receptor-mediated endocytosis, demanding high presentation of membrane-localized EphA2 receptors. For example, EphA2 agonistic monoclonal antibodies inhibit malignant cell behavior by promoting EphA2 receptor phosphorylation, internalization and subsequent degradation [43, 82]; EphA2 targeted immunoconjugates translocate therapeutic or radioactive agents into cells by EphA2 mediated endocytosis [84, 85]; adenovirus vector-mediated secretion of soluble EphrinA1-Fc elicits EphrinA1-EphA2 tumor inhibitory forward signaling followed by EphrinA1-EphA2 compound internalization [49]; small molecule inhibitors block the receptor and ligand interactions by occupying ligand binding domain in membrane-anchored EphA2 receptors [103].

However, along with the cell-penetrating peptides have emerged in recent years, plasma membrane localization of targets has not been considered a prerequisite for executing successful intracellular targeting [104]. Owning an excellent permeability and independence of plasma membrane receptors, cell penetrating peptides are able to directly penetrate the plasma membrane via an energy-independent pathway with high efficiency and subsequently bind to targets inside the targeted cells [105].

The high permeable cell-penetrating peptides provide a very convenient way for targeting molecules which attach themselves to the inner side of cells, thereby expanding the applicable scale of targeting therapy and imaging. The differential localization of EphA2 receptor in cells makes it possible to respond to a variety of targeting agents that alternatively target molecules either on plasma membrane or at the intracellular compartment. In this study it was demonstrated that EphA2 was overexpressed in three carcinomas at different locations. Esophageal adenocarcinoma and cardia adenocarcinoma overexpressed EphA2 at the intracellular part, while pancreatic adenocarcinoma overexpressed EphA2 on plasma membrane. Based on the above, we propose that an overexpression/localization-dependent pathway be seen as a rational foundation for choosing targeting agents for EphA2. For example, for carcinomas that overexpress EphA2 on the plasma membrane such as pancreatic adenocarcinoma, non-cell-penetrating EphA2-activating ligand peptides or antibodies would be the ideal agents for targeting, while for carcinomas overexpressing EphA2 inside cells such as esophageal adenocarcinoma and cardia adenocarcinoma, cell-penetrating peptides would be the most suitable candidates for targeting agents. Additionally, for carcinomas presenting overexpression of EphA2 at both sites, a combination of non-cell-penetrating EphA2-activating ligand peptide and cell-penetrating peptide seems to be the most effective targeting approach.

5.4 Correlations between EphA2 and tumor differentiation

Many studies investigated the correlation between EphA2 and clinicopathological parameters in a variety of tumors. The results vary across the type of tumor. Among them, a strong correlation was demonstrated between a high level of EphA2 expression and metastasis in cancers, including ovarian cancer, colorectal cancer, brain cancer, esophageal cancer and pancreatic cancer[106-110]. Least correlation was shown between the elevated EphA2 expression and the histological differentiation of lung cancer, stomach cancer, pancreatic cancer, colorectal cancer and esophageal squamous cell cancer[92, 106, 107, 111, 112]. With the limited information available, the correlation between EphA2 and the histological differentiation in our carcinoma samples was analyzed and found to be of no significance in any carcinoma, which was consistent with the results from other studies. However, rather uneven samples were involved in each kind of differentiation group, which may have possibly impacted the final statistical conclusion. A solution will require the extension of the sample size and a balanced sample in each differentiation group.

5.5 Perspective

The unique dual function of the EphA2 receptor in tumorigenesis and angiogenesis makes it a very attractive target. In general, there are at least two ways in which EphA2 can be targeted with respect to cancer therapy and diagnostics. The first involves taking advantage of the tumor-promoting function of EphA2 to modulate the behavior of tumor cells and suppress tumor growth and malignant progression. The second involves using the EphA2 receptor as a way of delivering agent to tumor cells and the associated vasculature in the form of either exogenous drugs or endogenous immune cells.

With regard to function-based EphA2 targeting, whether to pursue EphrinA1 ligand based therapeutics or take an approach that involves direct knockdown of EphA2 expression is rather a dilemma. Ligand based approaches, such as delivery of EphrinA1 ligand peptide to tumors, have the advantage of potentially inducing tumor-suppressive signaling through EphA2 in tumor cells, as well as further preventing the tumorigenic effects of EphA2 by down-regulating the receptor. On the other hand, one must exercise caution in view of the function of EphrinA1 ligand peptide in promoting angiogenesis through endothelial cell specific EphA2. For targeting tumor vasculature, the best approach may likely be to either interrupt the EphA2-EphrinA1 interaction by using a blocker such as EphA2-Fc or knockdown of EphA2 in endothelial cells. From this point of view, the most rational approach may be direct knockdown of EphA2 receptor expression that would potentially be effective in removing both the powerful oncoprotein from tumor cells and from tumor endothelial cells.

Another targeting scenario is to use the transmembrane-localized EphA2 receptor as a recognizing site for delivery of ligand-peptide based therapeutic or diagnostic agents to tumors. An advantage of these targeting agents is that any function elicited by the interactions between ligand peptides and EphA2 receptors is likely to be irrelevant, as the targeted cell is nearly immediately destined to die.

Although cell-penetrating peptides (CPPs) have a huge potential to translocate a variety of agents into the intracellular compartment, they still lack cell specificity which remains a major challenge for future applications in tumor molecular imaging and therapy. Indeed, most cells will internalize the CPP agent when they come in contact with it. As a consequence, the CPP agent will be taken up in the wrong cells and then increase normal cell toxicity severely and/or reduce imaging contrast significantly. Thus, as to targeting the intracellularly localized EphA2 receptor, efforts should be aimed first at elevating cell specificity.

6. Bibliography

1. Organization World Health. The Global Burden of Disease: 2004 Update.: Geneva: World Health Organization; 2008.
2. Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011,61:69-90.
3. Adams AB. Modern management of cancer patients: a multidisciplinary approach. Hosp Prog 1977,58:50, 52, 82.
4. Richard Pazdur LDW, Kevin A. Camphausen, William J. Hoskins. Cancer management: a multidisciplinary approach 13 ed: UBM Medica; 2011.
5. Strebhardt K, Ullrich A. Paul Ehrlich's magic bullet concept: 100 years of progress. Nat Rev Cancer 2008,8:473-480.
6. Schrama D, Reisfeld RA, Becker JC. Antibody targeted drugs as cancer therapeutics. Nat Rev Drug Discov 2006,5:147-159.
7. Trastuzumab. In: http://www.cancer.gov/cancertopics/druginfo/trastuzumab; October 5, 2006.
8. Rituximab. In: http://www.cancer.gov/cancertopics/druginfo/rituximab; October 5, 2006.
9. Bevacizumab. In: http://www.cancer.gov/cancertopics/druginfo/bevacizumab; October 5, 2006.
10. Serafini AN. From monoclonal antibodies to peptides and molecular recognition units: an overview. J Nucl Med 1993,34:533-536.
11. Behr TM, Memtsoudis S, Sharkey RM, et al. Experimental studies on the role of antibody fragments in cancer radio-immunotherapy: Influence of radiation dose and dose rate on toxicity and anti-tumor efficacy. Int J Cancer 1998,77:787-795.
12. Carter P. Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer 2001,1:118-129.
13. Pastan I, Hassan R, Fitzgerald DJ, et al. Immunotoxin therapy of cancer. Nat Rev Cancer 2006,6:559-565.
14. Pennell CA, Erickson HA. Designing immunotoxins for cancer therapy. Immunol Res 2002,25:177-191.

15. Khawli LA, Hu P, Epstein AL. Cytokine, chemokine, and co-stimulatory fusion proteins for the immunotherapy of solid tumors. Handb Exp Pharmacol 2008:291-328.

16. Goldenberg DM, Sharkey RM. Novel radiolabeled antibody conjugates. Oncogene 2007,26:3734-3744.

17. von Mehren M, Adams GP, Weiner LM. Monoclonal antibody therapy for cancer. Annu Rev Med 2003,54:343-369.

18. Bross PF, Beitz J, Chen G, et al. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. Clin Cancer Res 2001,7:1490-1496.

19. Sievers EL, Linenberger M. Mylotarg: antibody-targeted chemotherapy comes of age. Curr Opin Oncol 2001,13:522-527.

20. Arons E, Sorbara L, Raffeld M, et al. Characterization of T-cell repertoire in hairy cell leukemia patients before and after recombinant immunotoxin BL22 therapy. Cancer Immunol Immunother 2006,55:1100-1110.

21. Reubi JC. Neuropeptide receptors in health and disease: the molecular basis for in vivo imaging. J Nucl Med 1995,36:1825-1835.

22. Krenning EP, Bakker WH, Breeman WA, et al. Localisation of endocrine-related tumours with radioiodinated analogue of somatostatin. Lancet 1989,1:242-244.

23. Gibril F, Reynolds JC, Doppman JL, et al. Somatostatin receptor scintigraphy: its sensitivity compared with that of other imaging methods in detecting primary and metastatic gastrinomas. A prospective study. Ann Intern Med 1996,125:26-34.

24. Otte A, Mueller-Brand J, Dellas S, et al. Yttrium-90-labelled somatostatin-analogue for cancer treatment. Lancet 1998,351:417-418.

25. Waldherr C, Pless M, Maecke HR, et al. The clinical value of [90Y-DOTA]-D-Phe1-Tyr3-octreotide (90Y-DOTATOC) in the treatment of neuroendocrine tumours: a clinical phase II study. Ann Oncol 2001,12:941-945.

26. Paganelli G, Zoboli S, Cremonesi M, et al. Receptor-mediated radiotherapy with 90Y-DOTA-D-Phe1-Tyr3-octreotide. Eur J Nucl Med 2001,28:426-434.

27. Szepeshazi K, Schally AV, Halmos G, et al. Targeted cytotoxic somatostatin analogue AN-238 inhibits somatostatin receptor-positive experimental colon cancers independently of their p53 status. Cancer Res 2002,62:781-788.

28. Szereday Z, Schally AV, Szepeshazi K, et al. Effective treatment of H838 human non-small cell lung carcinoma with a targeted cytotoxic somatostatin analog, AN-238. Int J Oncol 2003,22:1141-1146.

29. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. Endocr Rev 2003,24:389-427.

30. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell 1990,61:203-212.

31. Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. Nat Rev Cancer 2004,4:361-370.

32. Lindberg RA, Hunter T. cDNA cloning and characterization of eck, an epithelial cell receptor protein-tyrosine kinase in the eph/elk family of protein kinases. Mol Cell Biol 1990,10:6316-6324.

33. Ruiz JC, Robertson EJ. The expression of the receptor-protein tyrosine kinase gene, eck, is highly restricted during early mouse development. Mech Dev 1994,46:87-100.

34. Himanen JP, Goldgur Y, Miao H, et al. Ligand recognition by A-class Eph receptors: crystal structures of the EphA2 ligand-binding domain and the EphA2/ephrin-A1 complex. EMBO Rep 2009,10:722-728.

35. Shao H, Pandey A, O'Shea KS, et al, Dixit VM. Characterization of B61, the ligand for the Eck receptor protein-tyrosine kinase. J Biol Chem 1995,270:5636-5641.

36. Holzman LB, Marks RM, et al. A novel immediate-early response gene of endothelium is induced by cytokines and encodes a secreted protein. Mol Cell Biol 1990,10:5830-5838.

37. Murai KK, Pasquale EB. 'Eph'ective signaling: forward, reverse and crosstalk. J Cell Sci 2003,116:2823-2832.

38. Kullander K, Klein R. Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol 2002,3:475-486.

39. Noren NK, Pasquale EB. Eph receptor-ephrin bidirectional signals that target Ras and Rho proteins. Cell Signal 2004,16:655-666.

40. Tandon M, Vemula SV, Mittal SK. Emerging strategies for EphA2 receptor targeting for cancer therapeutics. Expert Opin Ther Targets 2011,15:31-51.

41. Zantek ND, Azimi M, Fedor-Chaiken M, et al. E-cadherin regulates the function of the

EphA2 receptor tyrosine kinase. Cell Growth Differ 1999,10:629-638.

42. Zelinski DP, Zantek ND, Stewart JC, et al. EphA2 overexpression causes tumorigenesis of mammary epithelial cells. Cancer Res 2001,61:2301-2306.

43. Carles-Kinch K, Kilpatrick KE, Stewart JC, et al. Antibody targeting of the EphA2 tyrosine kinase inhibits malignant cell behavior. Cancer Res 2002,62:2840-2847.

44. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer 2010,10:165-180.

45. Pitulescu ME, Adams RH. Eph/ephrin molecules--a hub for signaling and endocytosis. Genes Dev 2010,24:2480-2492.

46. Heroult M, Schaffner F, Augustin HG. Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. Exp Cell Res 2006,312:642-650.

47. Cheng N, Brantley DM, Chen J. The ephrins and Eph receptors in angiogenesis. Cytokine Growth Factor Rev 2002,13:75-85.

48. Pasquale EB. Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol 2005,6:462-475.

49. Noblitt LW, Bangari DS, Shukla S, et al. Decreased tumorigenic potential of EphA2-overexpressing breast cancer cells following treatment with adenoviral vectors that express EphrinA1. Cancer Gene Ther 2004,11:757-766.

50. Miao H, Burnett E, Kinch M, et al. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. Nat Cell Biol 2000,2:62-69.

51. Kinch MS, Carles-Kinch K. Overexpression and functional alterations of the EphA2 tyrosine kinase in cancer. Clin Exp Metastasis 2003,20:59-68.

52. Walker-Daniels J, Hess AR, Hendrix MJ, et al. Differential regulation of EphA2 in normal and malignant cells. Am J Pathol 2003,162:1037-1042.

53. Larsen AB, Pedersen MW, Stockhausen MT, et al. Activation of the EGFR gene target EphA2 inhibits epidermal growth factor-induced cancer cell motility. Mol Cancer Res 2007,5:283-293.

54. Brantley-Sieders DM, Zhuang G, Hicks D, et al. The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice

by amplifying ErbB2 signaling. J Clin Invest 2008,118:64-78.

55. Miao H, Li DQ, Mukherjee A, et al. EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. Cancer Cell 2009,16:9-20.

56. Vaught D, Chen J, Brantley-Sieders DM. Regulation of mammary gland branching morphogenesis by EphA2 receptor tyrosine kinase. Mol Biol Cell 2009,20:2572-2581.

57. Walker-Daniels J, Coffman K, Azimi M, et al. Overexpression of the EphA2 tyrosine kinase in prostate cancer. Prostate 1999,41:275-280.

58. D'Amico TA, Aloia TA, Moore MB, et al. Predicting the sites of metastases from lung cancer using molecular biologic markers. Ann Thorac Surg 2001,72:1144-1148.

59. Easty DJ, Bennett DC. Protein tyrosine kinases in malignant melanoma. Melanoma Res 2000,10:401-411.

60. Thaker PH, Deavers M, Celestino J, et al. EphA2 expression is associated with aggressive features in ovarian carcinoma. Clin Cancer Res 2004,10:5145-5150.

61. Herath NI, Boyd AW. The role of Eph receptors and ephrin ligands in colorectal cancer. Int J Cancer 2010,126:2003-2011.

62. Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell 2008,133:38-52.

63. Fang WB, Ireton RC, Zhuang G, et al. Overexpression of EPHA2 receptor destabilizes adherens junctions via a RhoA-dependent mechanism. J Cell Sci 2008,121:358-368.

64. Duxbury MS, Ito H, Zinner MJ, et al. EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma. Oncogene 2004,23:1448-1456.

65. Landen CN, Jr., Chavez-Reyes A, Bucana C, et al. Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. Cancer Res 2005,65:6910-6918.

66. Duxbury MS, Ito H, Zinner MJ, et al. Ligation of EphA2 by Ephrin A1-Fc inhibits pancreatic adenocarcinoma cellular invasiveness. Biochem Biophys Res Commun 2004,320:1096-1102.

67. Zhang Y, Thant AA, Hiraiwa Y, et al. A role for focal adhesion kinase in

hyluronan-dependent MMP-2 secretion in a human small-cell lung carcinoma cell line, QG90. Biochem Biophys Res Commun 2002,290:1123-1127.

68. Sein TT, Thant AA, Hiraiwa Y, et al. A role for FAK in the Concanavalin A-dependent secretion of matrix metalloproteinase-2 and -9. Oncogene 2000,19:5539-5542.

69. Leroy C, Fialin C, Sirvent A, et al. Quantitative phosphoproteomics reveals a cluster of tyrosine kinases that mediates SRC invasive activity in advanced colon carcinoma cells. Cancer Res 2009,69:2279-2286.

70. Fang WB, Brantley-Sieders DM, Parker MA, et al. A kinase-dependent role for EphA2 receptor in promoting tumor growth and metastasis. Oncogene 2005,24:7859-7868.

71. Parri M, Taddei ML, Bianchini F, et al. EphA2 reexpression prompts invasion of melanoma cells shifting from mesenchymal to amoeboid-like motility style. Cancer Res 2009,69:2072-2081.

72. Zhuang G, Brantley-Sieders DM, Vaught D, et al. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. Cancer Res 2010,70:299-308.

73. Ogawa K, Pasqualini R, Lindberg RA, et al. The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. Oncogene 2000,19:6043-6052.

74. Cheng N, Brantley DM, Liu H, et al. Blockade of EphA receptor tyrosine kinase activation inhibits vascular endothelial cell growth factor-induced angiogenesis. Mol Cancer Res 2002,1:2-11.

75. Brantley DM, Cheng N, Thompson EJ, et al. Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. Oncogene 2002,21:7011-7026.

76. Dobrzanski P, Hunter K, Jones-Bolin S, et al. Antiangiogenic and antitumor efficacy of EphA2 receptor antagonist. Cancer Res 2004,64:910-919.

77. Brantley-Sieders DM, Fang WB, Hicks DJ, et al. Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression. FASEB J 2005,19:1884-1886.

78. Brantley-Sieders DM, Fang WB, Hwang Y, et al. Ephrin-A1 facilitates mammary tumor metastasis through an angiogenesis-dependent mechanism mediated by EphA receptor and vascular endothelial growth factor in mice. Cancer Res 2006,66:10315-10324.

79. Zhou Z, Yuan X, Li Z, et al. RNA interference targeting EphA2 inhibits proliferation,

induces apoptosis, and cooperates with cytotoxic drugs in human glioma cells. Surg Neurol 2008,70:562-568; discussion 568-569.

80. Wesa AK, Herrem CJ, Mandic M, et al. Enhancement in specific CD8+ T cell recognition of EphA2+ tumors in vitro and in vivo after treatment with ligand agonists. J Immunol 2008,181:7721-7727.

81. Bruckheimer EM, Fazenbaker CA, Gallagher S, et al. Antibody-dependent cell-mediated cytotoxicity effector-enhanced EphA2 agonist monoclonal antibody demonstrates potent activity against human tumors. Neoplasia 2009,11:509-517, 502 p following 517.

82. Landen CN, Jr., Lu C, Han LY, et al. Efficacy and antivascular effects of EphA2 reduction with an agonistic antibody in ovarian cancer. J Natl Cancer Inst 2006,98:1558-1570.

83. Hammond SA, Lutterbuese R, Roff S, et al. Selective targeting and potent control of tumor growth using an EphA2/CD3-Bispecific single-chain antibody construct. Cancer Res 2007,67:3927-3935.

84. Lee JW, Han HD, Shahzad MM, et al. EphA2 immunoconjugate as molecularly targeted chemotherapy for ovarian carcinoma. J Natl Cancer Inst 2009,101:1193-1205.

85. Cai W, Ebrahimnejad A, Chen K, et al. Quantitative radioimmunoPET imaging of EphA2 in tumor-bearing mice. Eur J Nucl Med Mol Imaging 2007,34:2024-2036.

86. Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathologe 1987,8:138-140.

87. Macrae M, Neve RM, Rodriguez-Viciana P, et al. A conditional feedback loop regulates Ras activity through EphA2. Cancer Cell 2005,8:111-118.

88. Pratt RL, Kinch MS. Ligand binding up-regulates EphA2 messenger RNA through the mitogen-activated protein/extracellular signal-regulated kinase pathway. Mol Cancer Res 2003,1:1070-1076.

89. Larsen AB, Stockhausen MT, Poulsen HS. Cell adhesion and EGFR activation regulate EphA2 expression in cancer. Cell Signal 2010,22:636-644.

90. Dohn M, Jiang J, Chen X. Receptor tyrosine kinase EphA2 is regulated by p53-family proteins and induces apoptosis. Oncogene 2001,20:6503-6515.

91. Zelinski DP, Zantek ND, Walker-Daniels J, et al. Estrogen and Myc negatively regulate expression of the EphA2 tyrosine kinase. J Cell Biochem 2002,85:714-720.

92. Mudali SV, Fu B, Lakkur SS, et al. Patterns of EphA2 protein expression in primary and metastatic pancreatic carcinoma and correlation with genetic status. Clin Exp Metastasis 2006,23:357-365.

93. Hafner C, Becker B, Landthaler M, et al. Expression profile of Eph receptors and ephrin ligands in human skin and downregulation of EphA1 in nonmelanoma skin cancer. Mod Pathol 2006,19:1369-1377.

94. Herath NI, Doecke J, Spanevello MD, et al. Epigenetic silencing of EphA1 expression in colorectal cancer is correlated with poor survival. Br J Cancer 2009,100:1095-1102.

95. Muller-Tidow C, Diederichs S, Bulk E, et al. Identification of metastasis-associated receptor tyrosine kinases in non-small cell lung cancer. Cancer Res 2005,65:1778-1782.

96. Batlle E, Bacani J, Begthel H, et al. EphB receptor activity suppresses colorectal cancer progression. Nature 2005,435:1126-1130.

97. Alazzouzi H, Davalos V, Kokko A, et al. Mechanisms of inactivation of the receptor tyrosine kinase EPHB2 in colorectal tumors. Cancer Res 2005,65:10170-10173.

98. Davalos V, Dopeso H, Castano J, et al. EPHB4 and survival of colorectal cancer patients. Cancer Res 2006,66:8943-8948.

99. Fu T, Li P, Wang H, et al. c-Rel is a transcriptional repressor of EPHB2 in colorectal cancer. J Pathol 2009,219:103-113.

100. Hafner C, Schmitz G, Meyer S, et al. Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers. Clin Chem 2004,50:490-499.

101. Brantley-Sieders DM, Caughron J, Hicks D, et al. EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation. J Cell Sci 2004,117:2037-2049.

102. Hess AR, Seftor EA, Gardner LM, et al. Molecular regulation of tumor cell vasculogenic mimicry by tyrosine phosphorylation: role of epithelial cell kinase (Eck/EphA2). Cancer Res 2001,61:3250-3255.

103. Noberini R, Koolpe M, Peddibhotla S, et al. Small molecules can selectively inhibit ephrin binding to the EphA4 and EphA2 receptors. J Biol Chem 2008,283:29461-29472.

104. Nasrollahi SA, Taghibiglou C, Azizi E, et al. Cell-penetrating Peptides as a Novel Transdermal Drug Delivery System. Chem Biol Drug Des 2012.

105. Madani F, Lindberg S, Langel U, et al. Mechanisms of cellular uptake of cell-penetrating peptides. J Biophys 2011,2011:414729.

106. Kinch MS, Moore MB, Harpole DH, Jr. Predictive value of the EphA2 receptor tyrosine kinase in lung cancer recurrence and survival. Clin Cancer Res 2003,9:613-618.

107. Miyazaki T, Kato H, Fukuchi M, et al. EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. Int J Cancer 2003,103:657-663.

108. Saito T, Masuda N, Miyazaki T, et al. Expression of EphA2 and E-cadherin in colorectal cancer: correlation with cancer metastasis. Oncol Rep 2004,11:605-611.

109. Lu C, Shahzad MM, Wang H, et al. EphA2 overexpression promotes ovarian cancer growth. Cancer Biol Ther 2008,7:1098-1103.

110. Yachida S, Iacobuzio-Donahue CA. The pathology and genetics of metastatic pancreatic cancer. Arch Pathol Lab Med 2009,133:413-422.

111. Kataoka H, Igarashi H, Kanamori M, et al. Correlation of EPHA2 overexpression with high microvessel count in human primary colorectal cancer. Cancer Sci 2004,95:136-141.

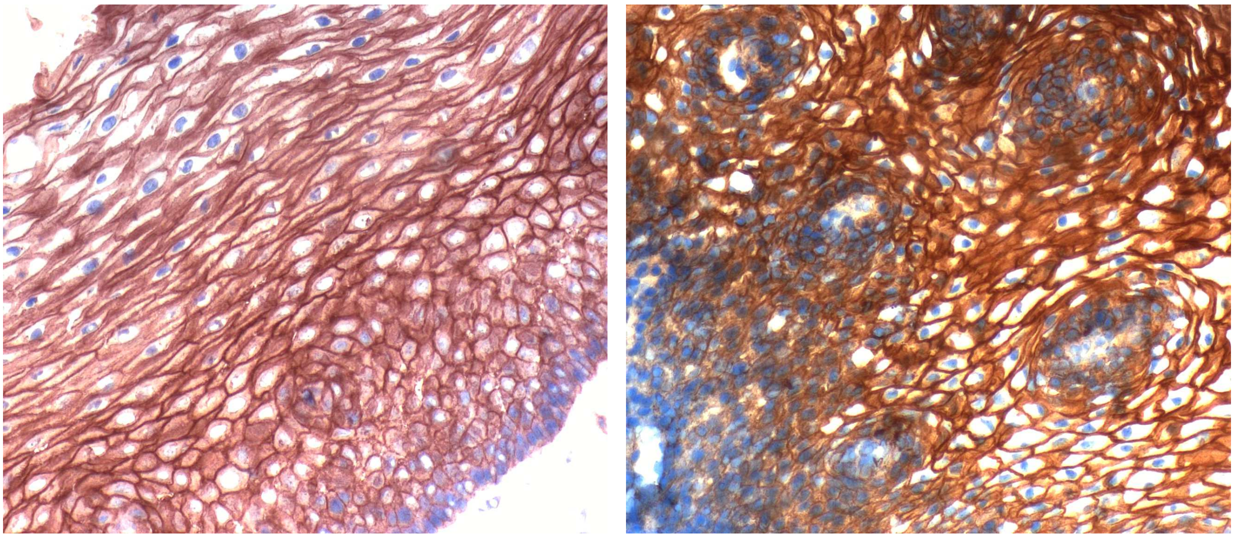
112. Yuan W, Chen Z, Wu S, et al. Expression of EphA2 and E-cadherin in gastric cancer: correlated with tumor progression and lymphogenous metastasis. Pathol Oncol Res 2009,15:473-478.

7. Appendix

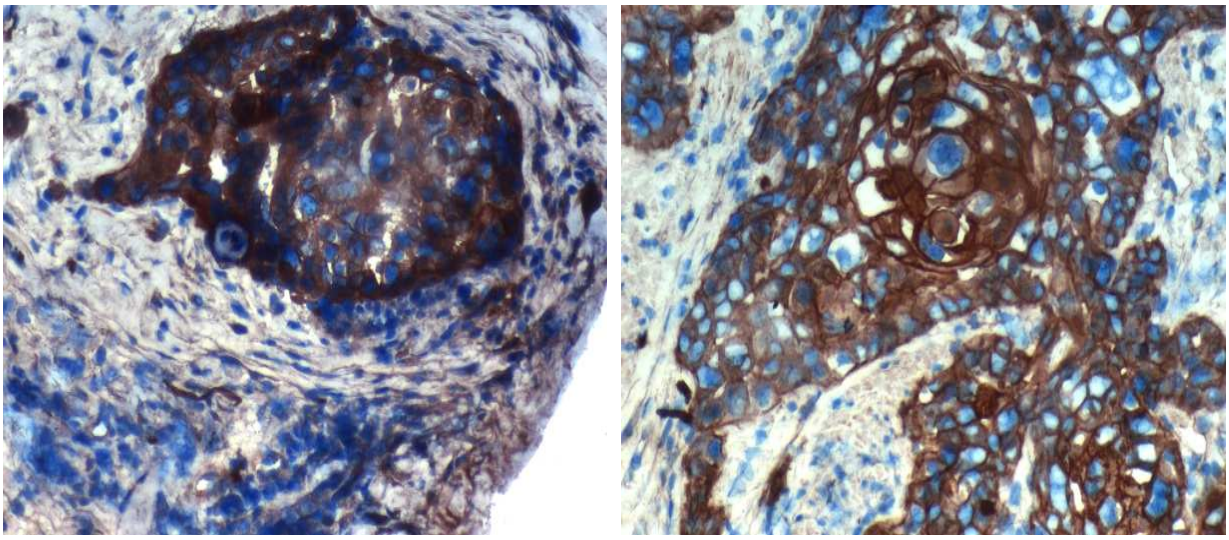
7.1 Immunohistochemial staining pictures of snap frozen tissues

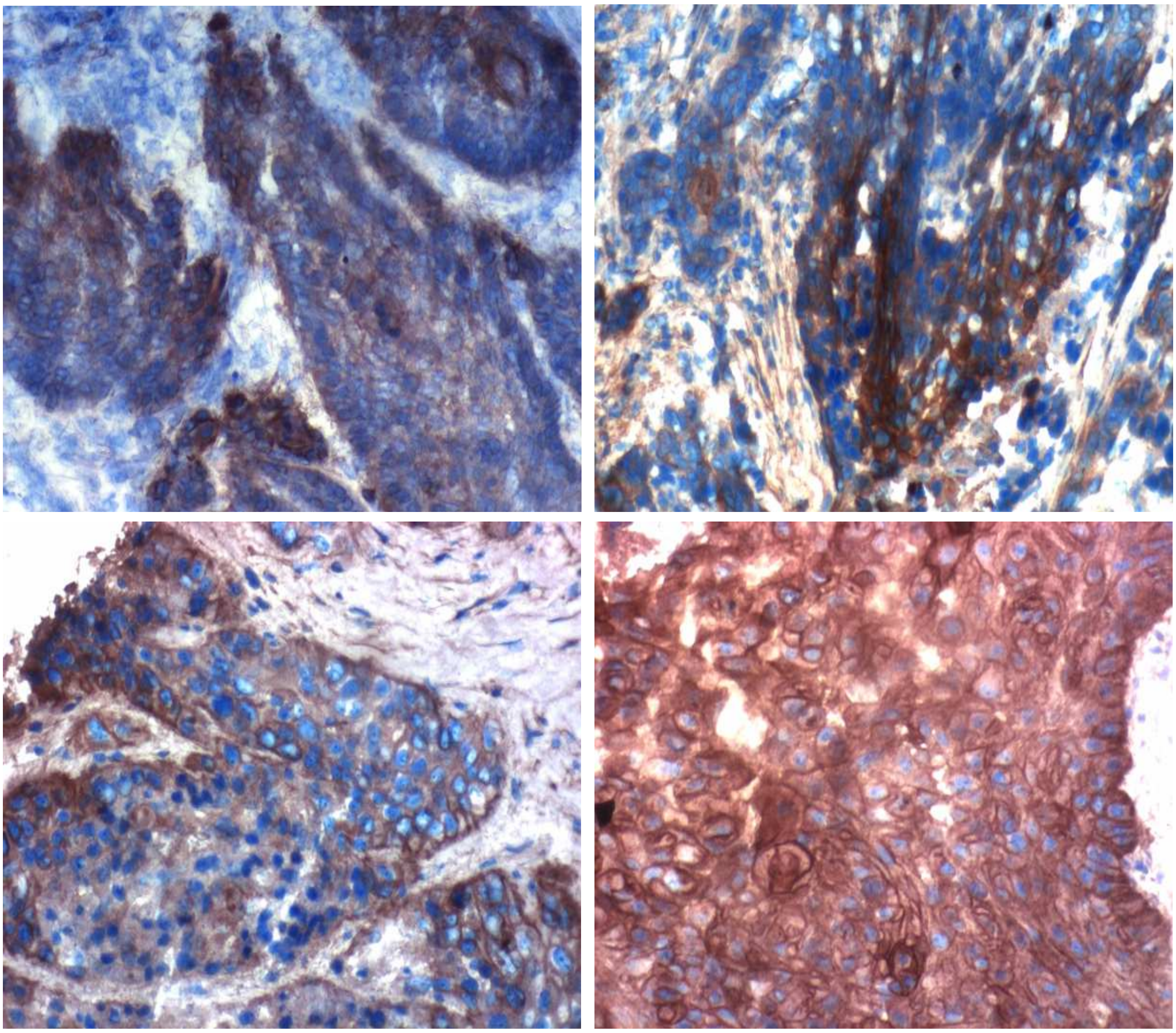
7.1.1 Esophagus

7.1.1.1 Normal esophagus

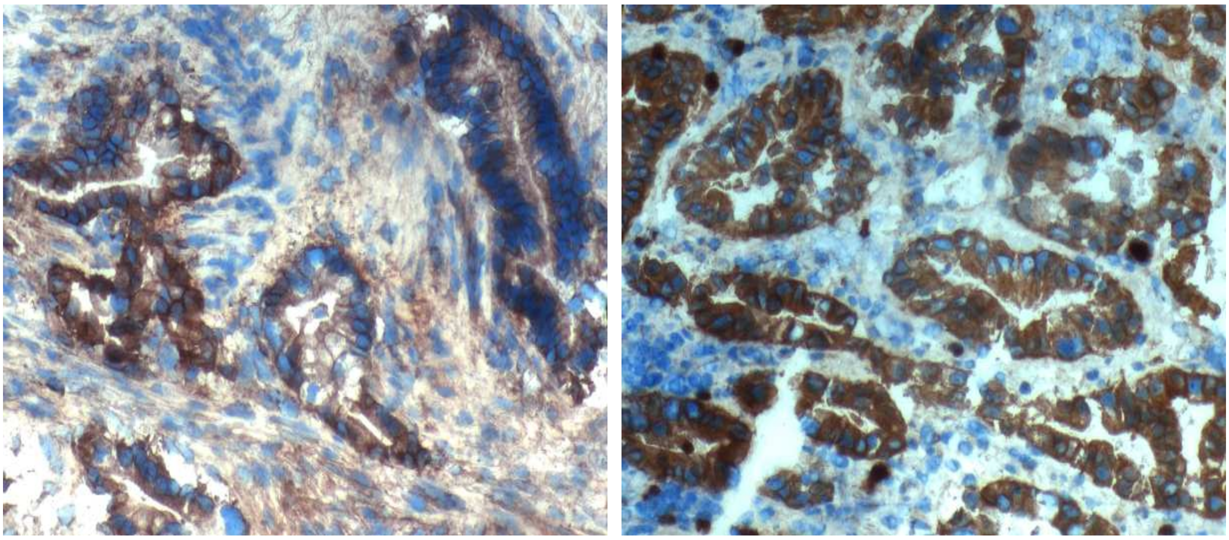


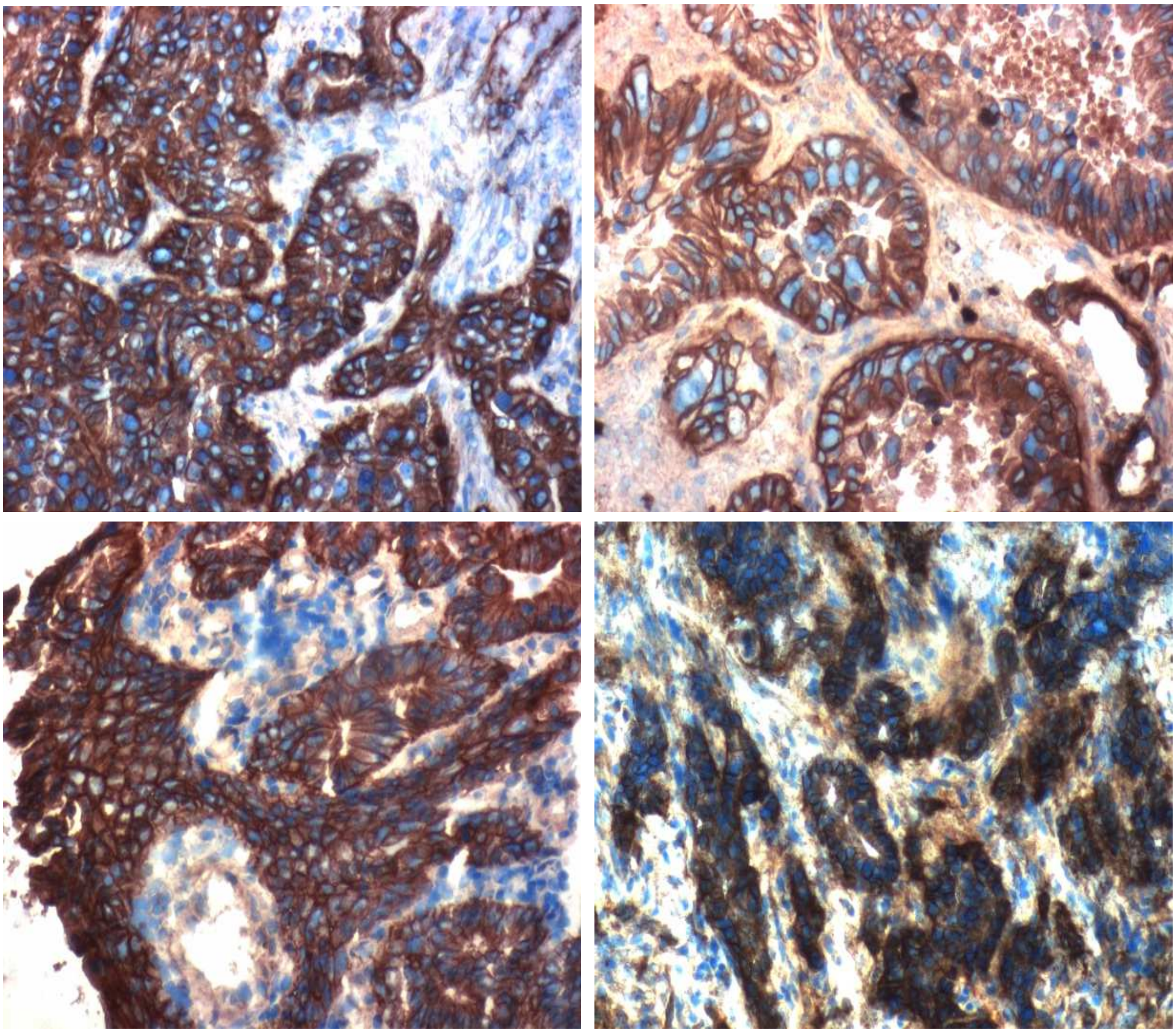
7.1.1.2 Esophageal squamous cell carcinoma





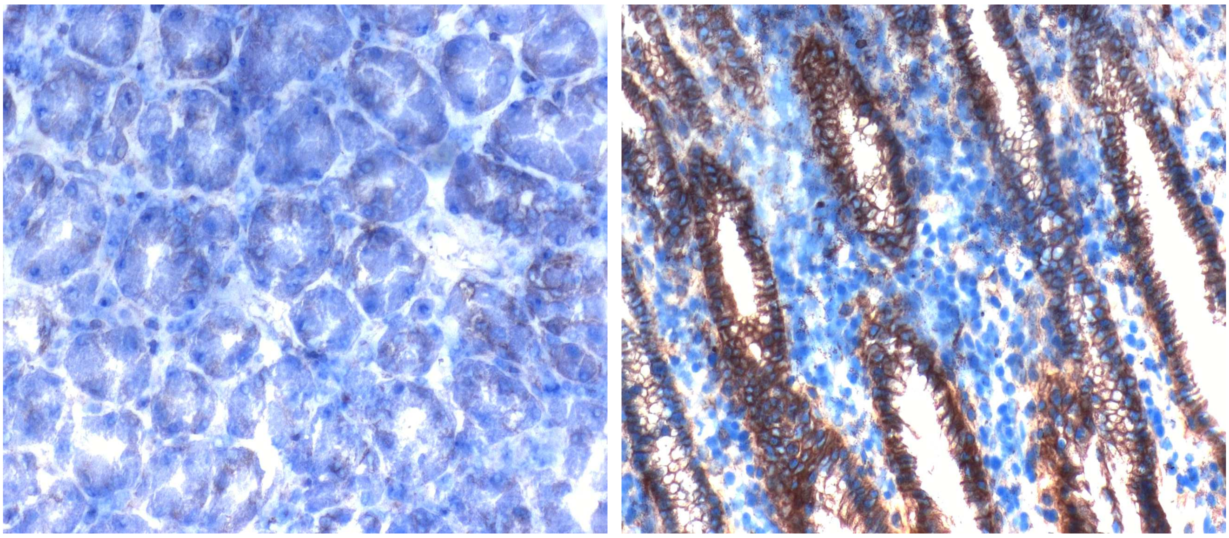
7.1.1.3 Esophageal adenocarcinoma



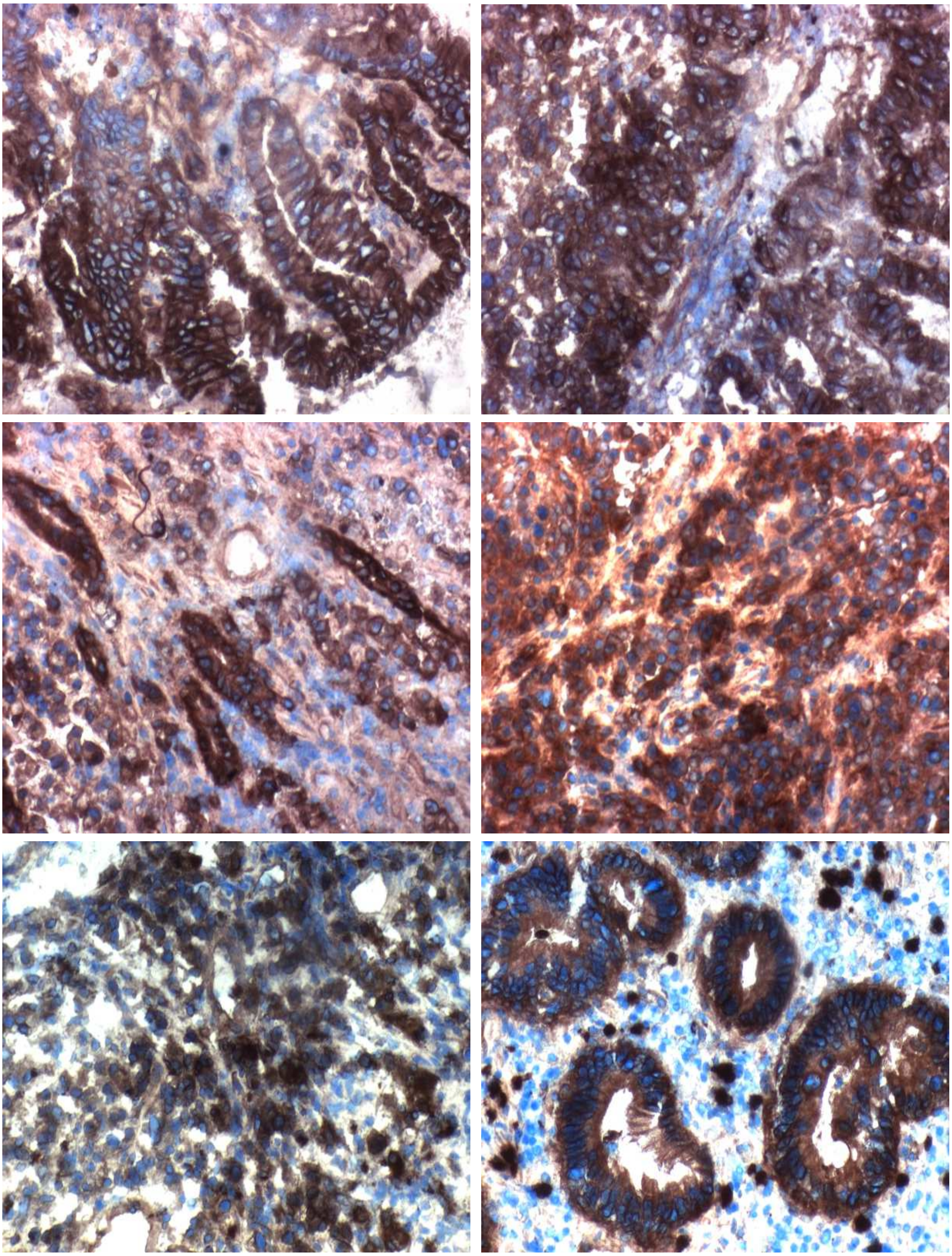


7.1.2 Stomach

7.1.2.1 Normal stomach

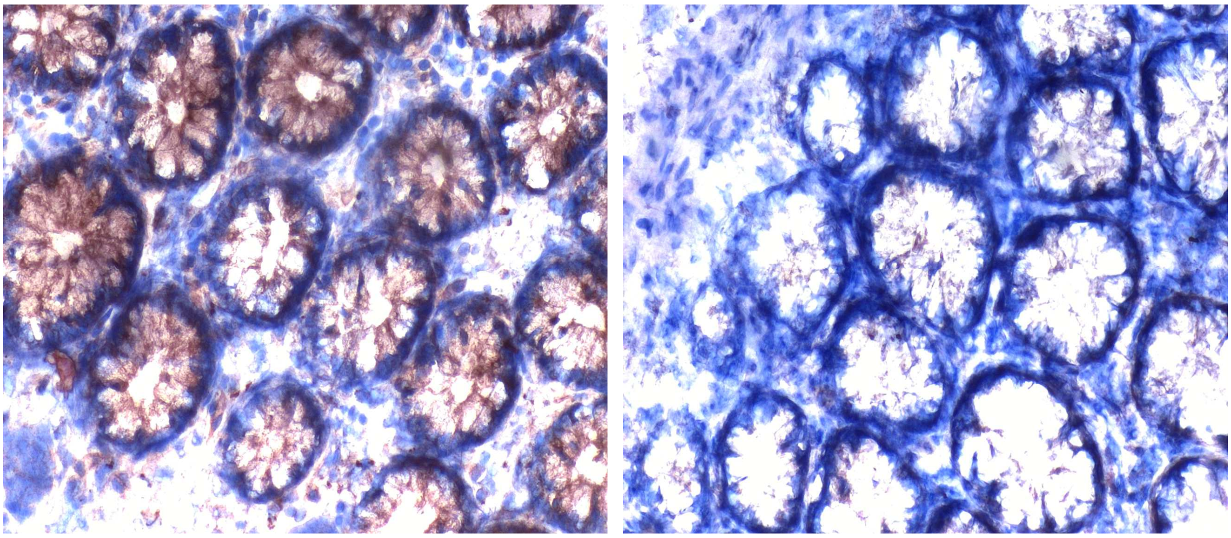


7.1.2.2 Stomach adenocarcinoma

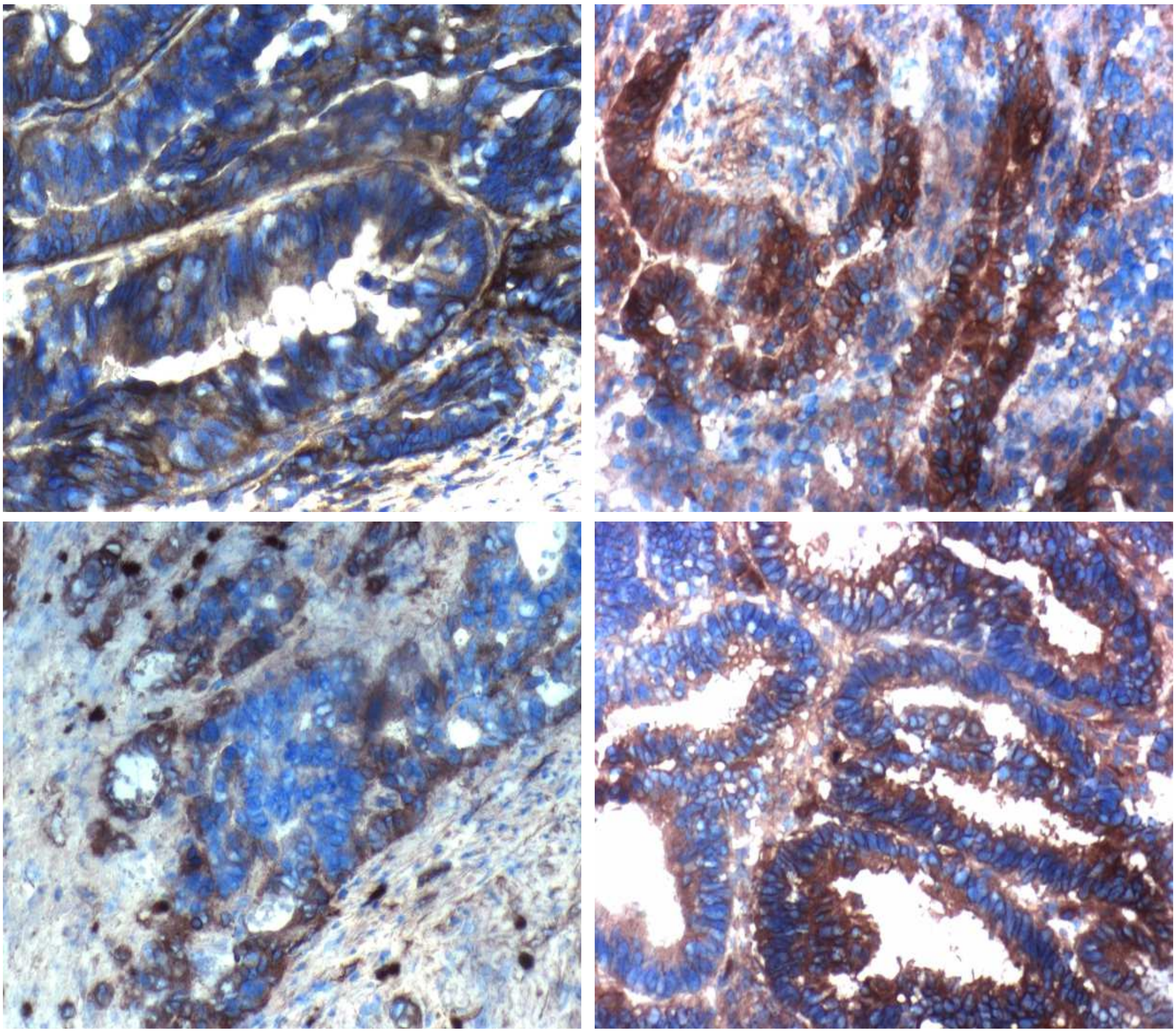


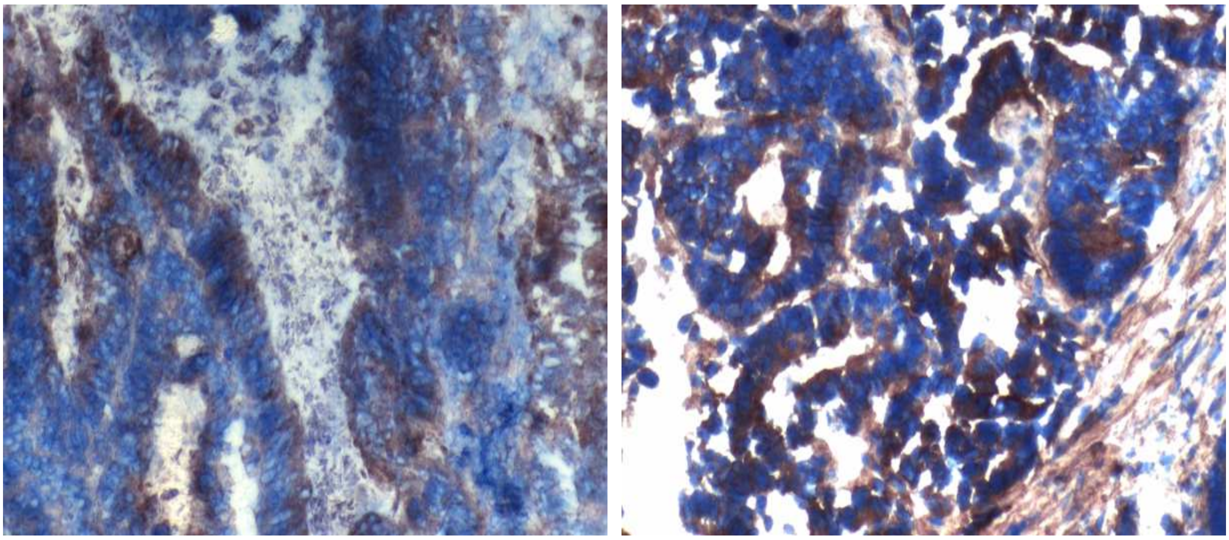
7.1.3 Colon-rectum

7.1.3.1 Normal colon-rectum



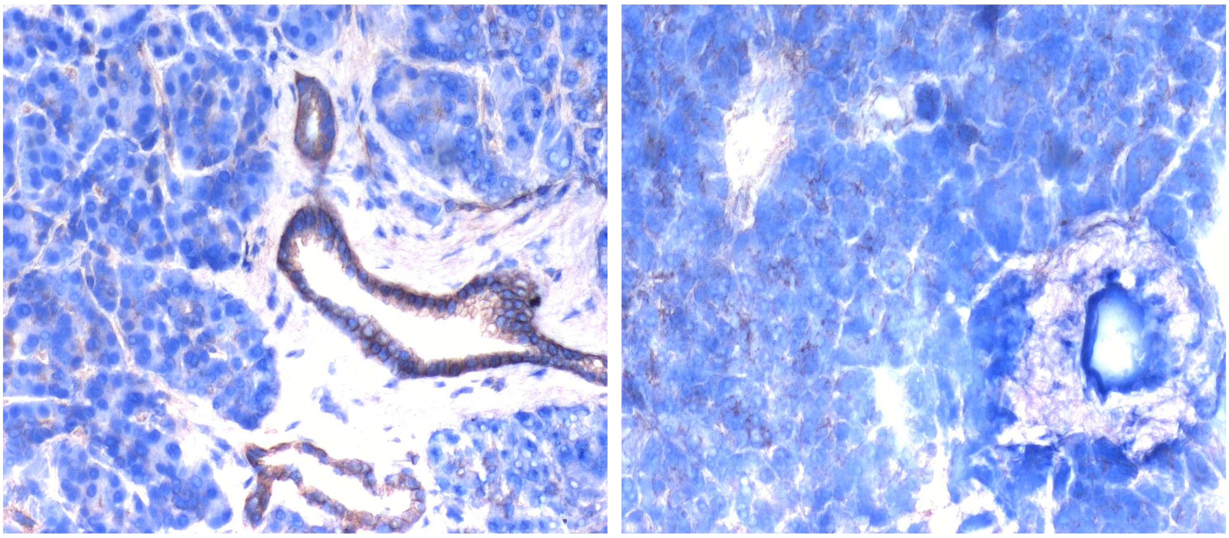
7.1.3.2 Colorectal adenocarcinoma



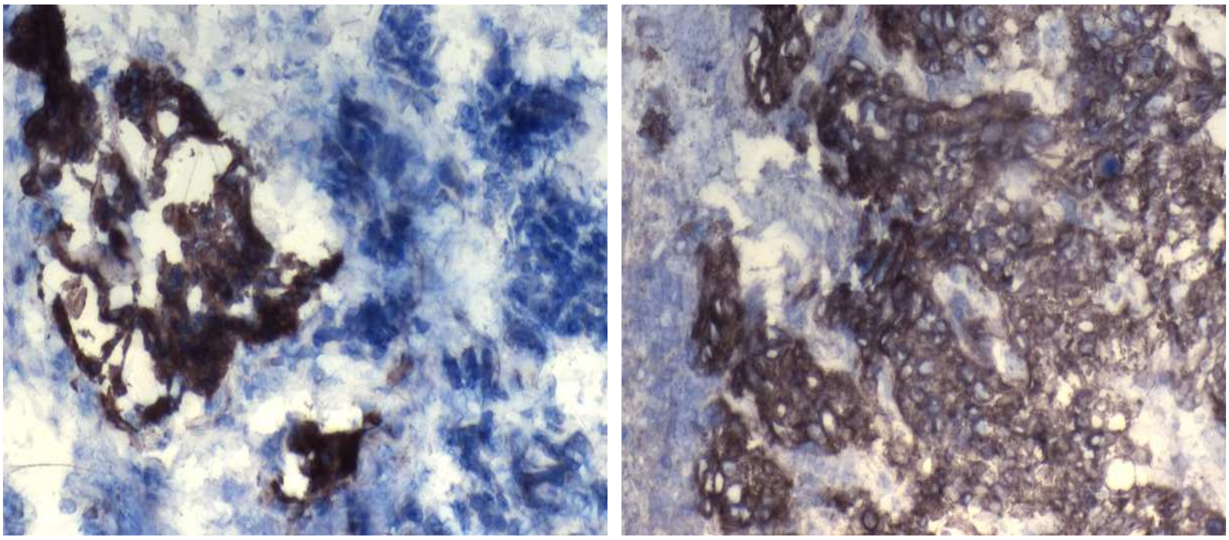


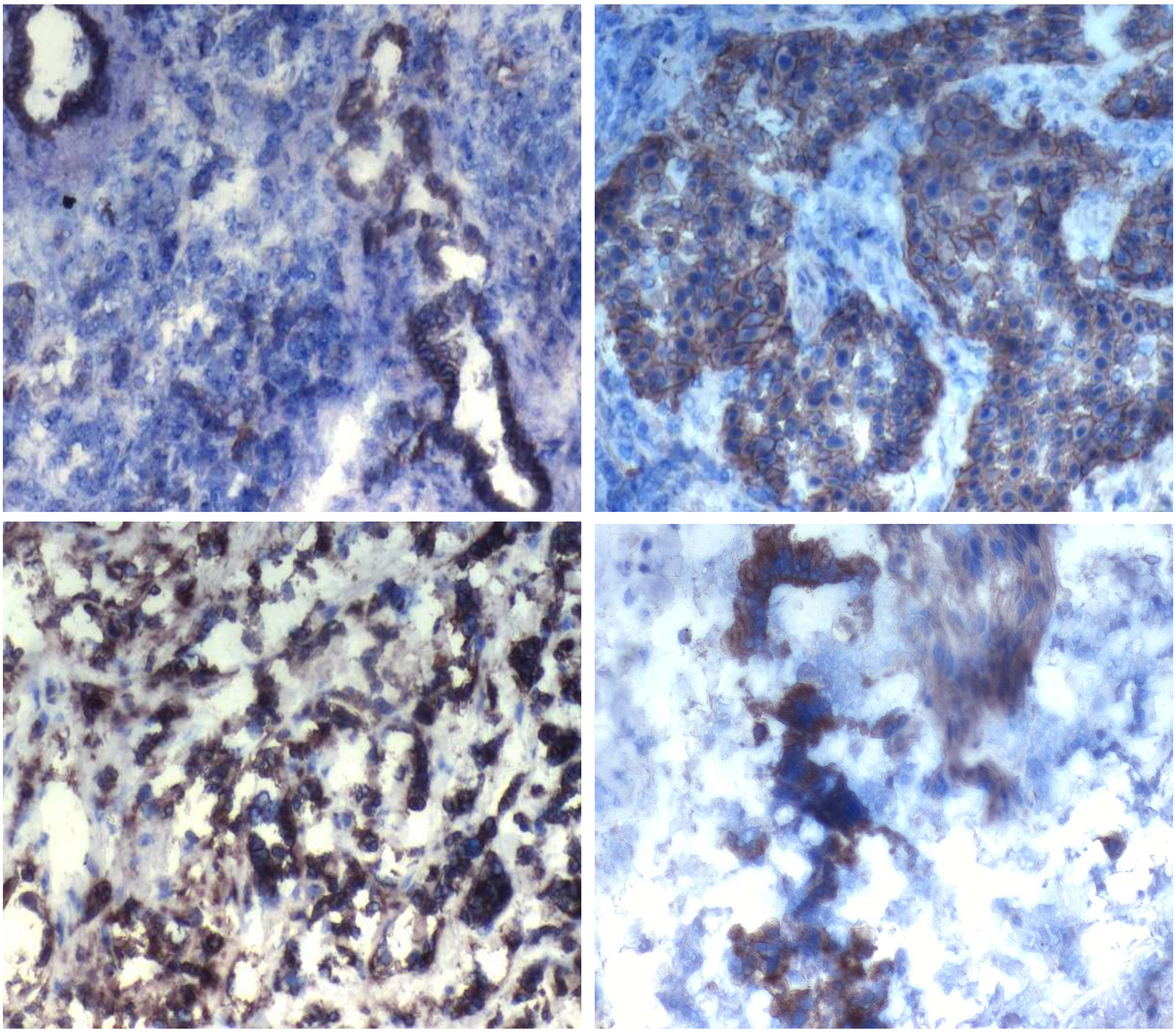
7.1.4 Pancreas

7.1.4.1 Normal pancreas



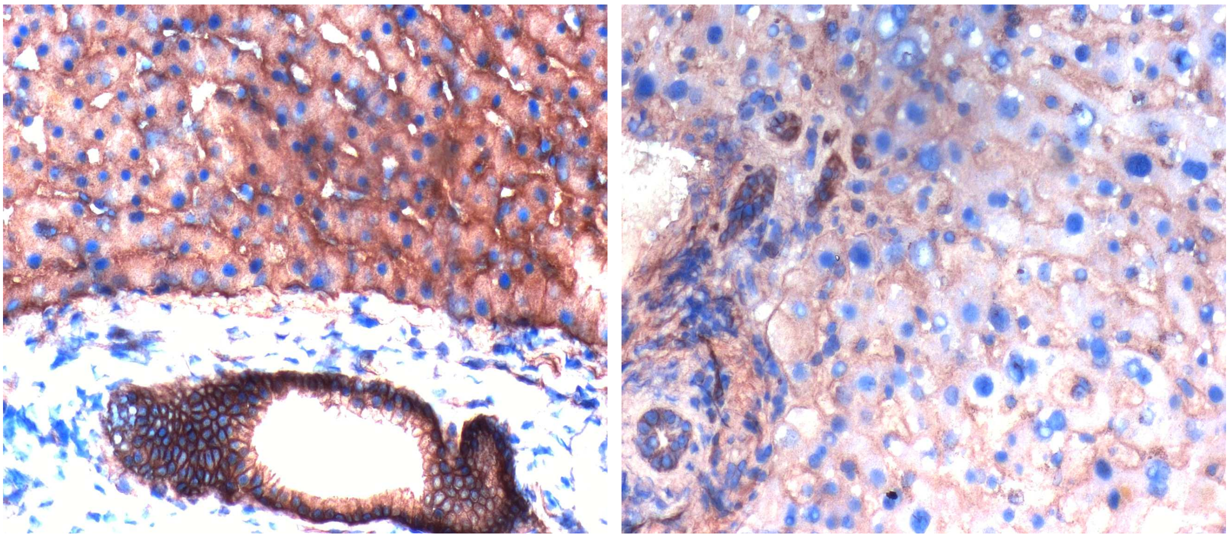
7.1.4.2 Pancreatic adenocarcinoma



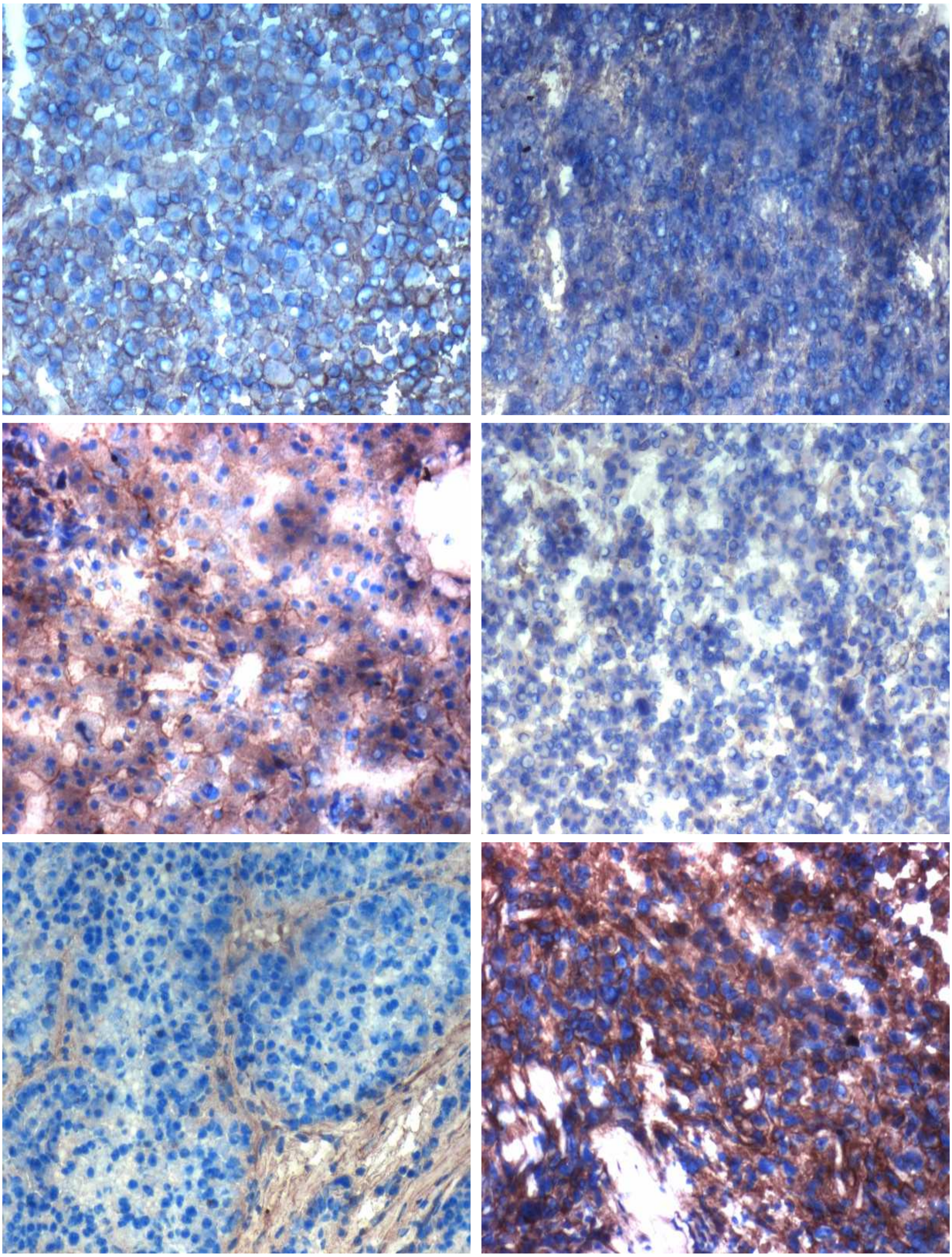


7.1.5 Liver

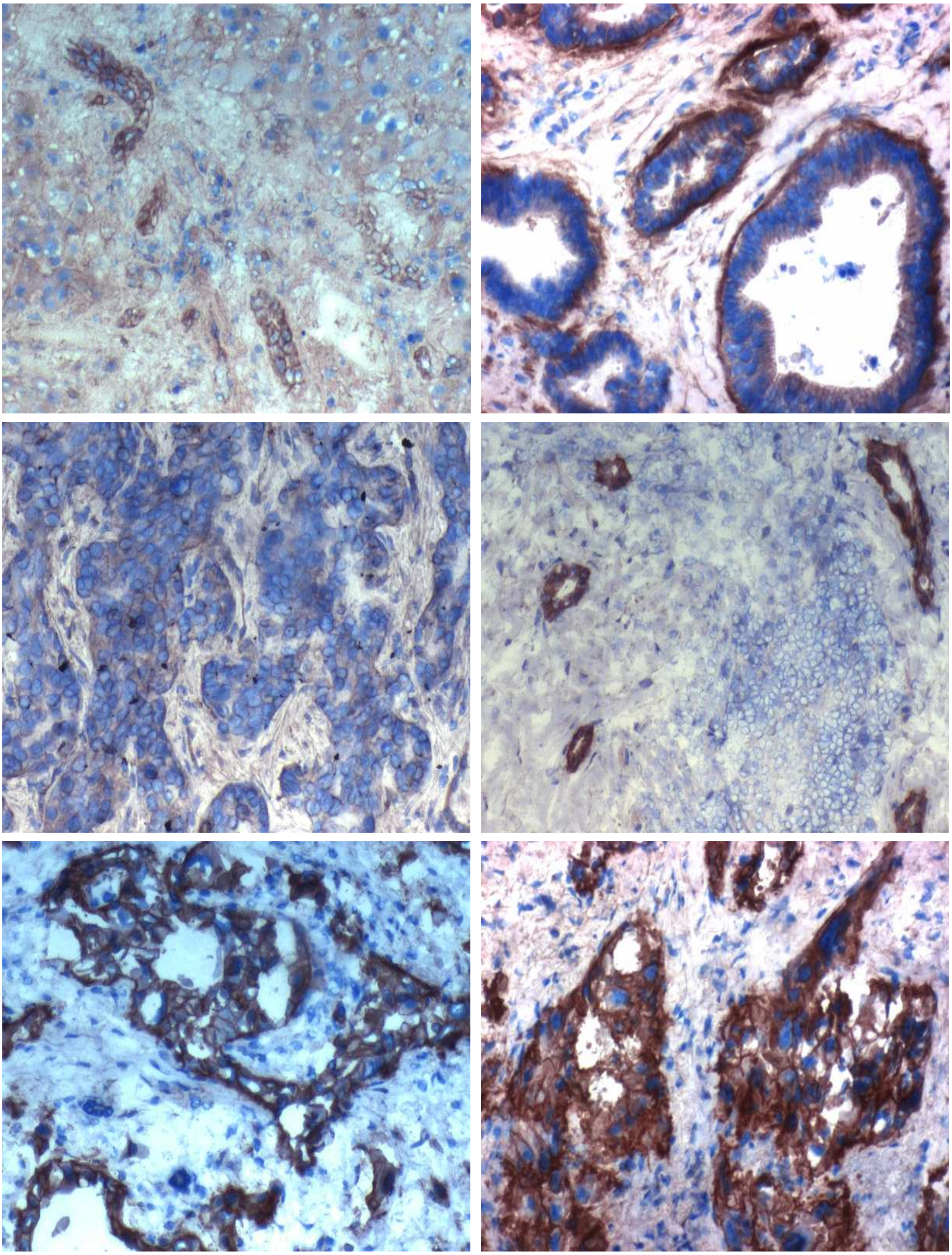
7.1.5.1 Normal liver



7.1.5.2 Hepatocellular carcinoma



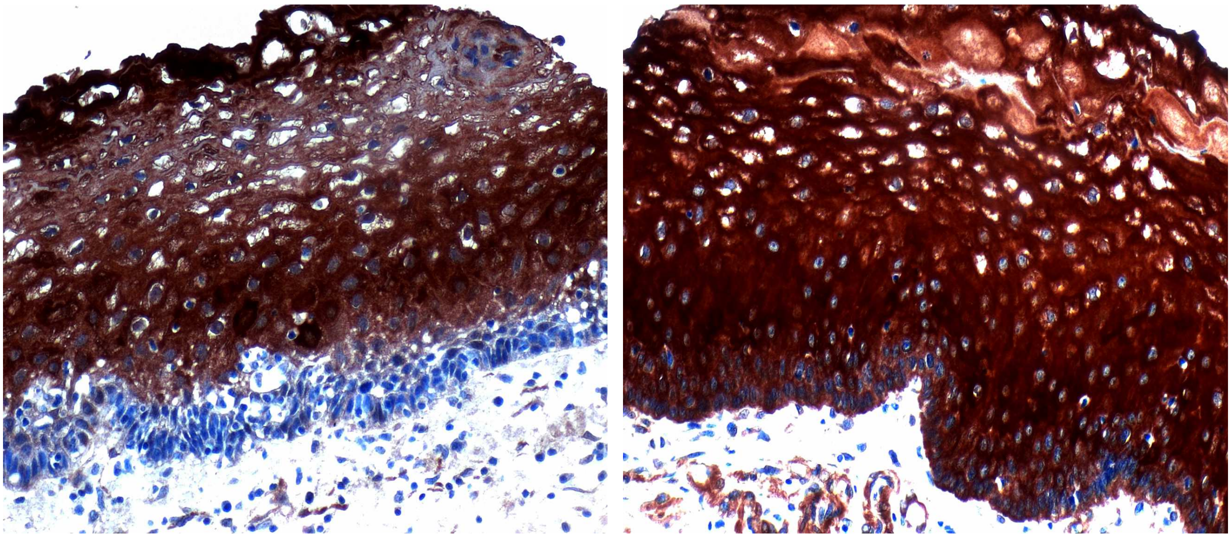
7.1.5.3 Cholangiocarcinoma



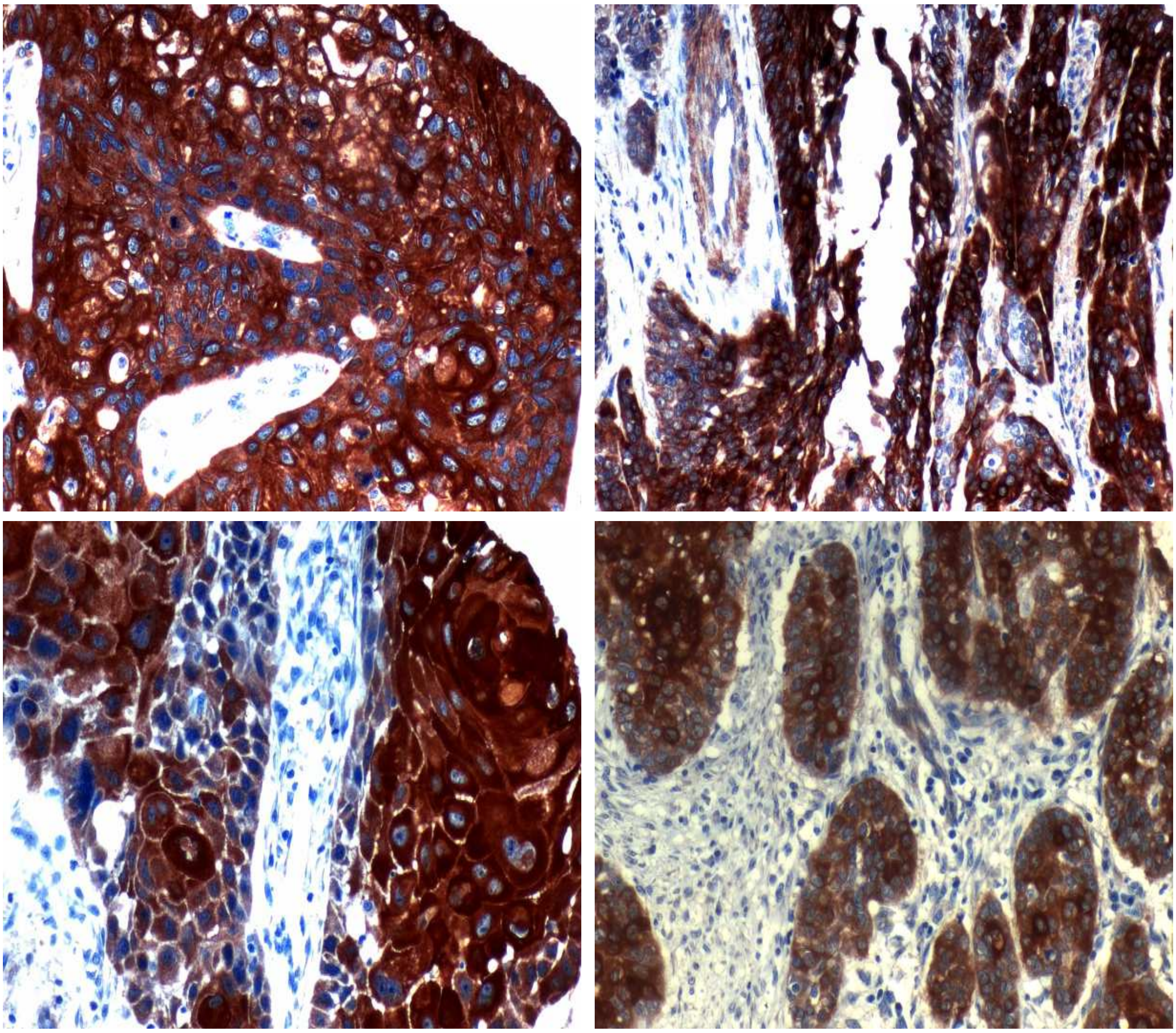
7.2 Immunohistochemical staining pictures of formalin fixed paraffin embeded tissue micro arrays (TMA)

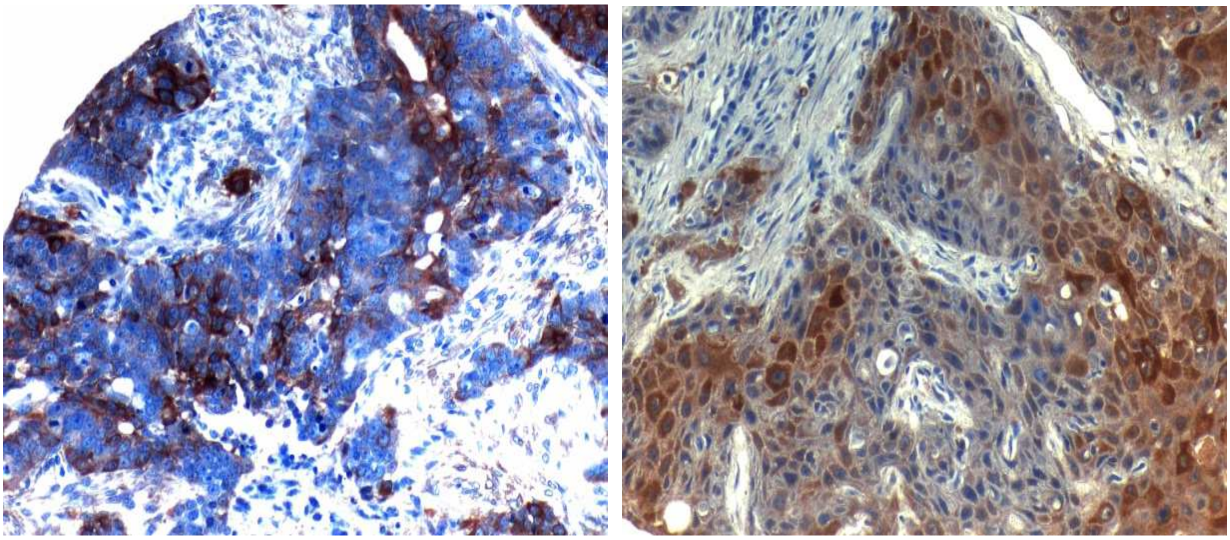
7.2.1 Esophagus

7.2.1.1 Normal esophagus (corresponding to the TMA slide of esophageal squamous cell carcinoma)

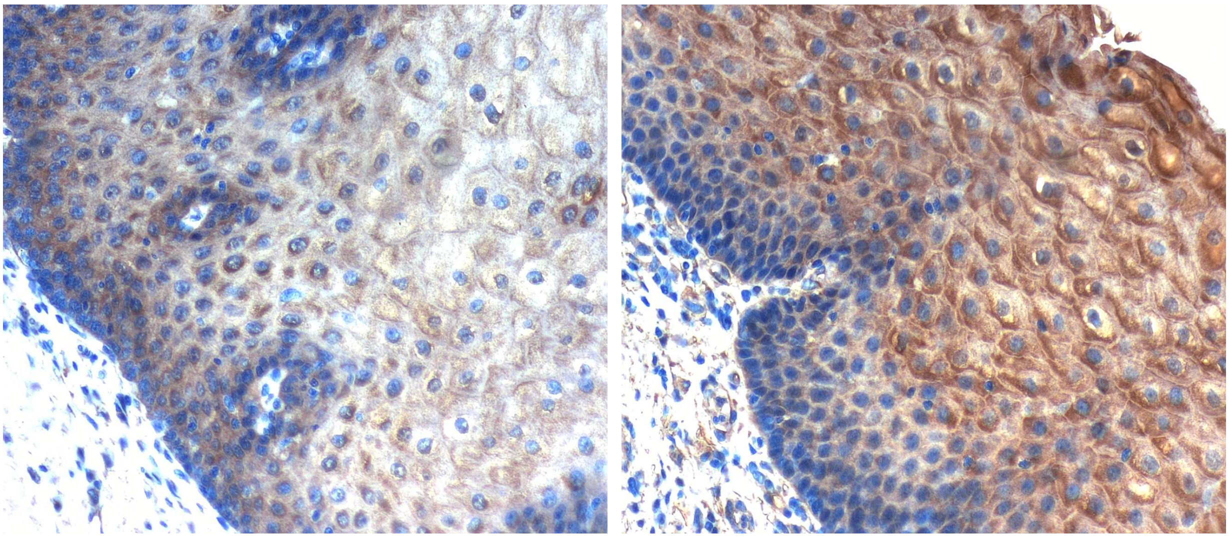


Esophageal squamous cell carcinoma

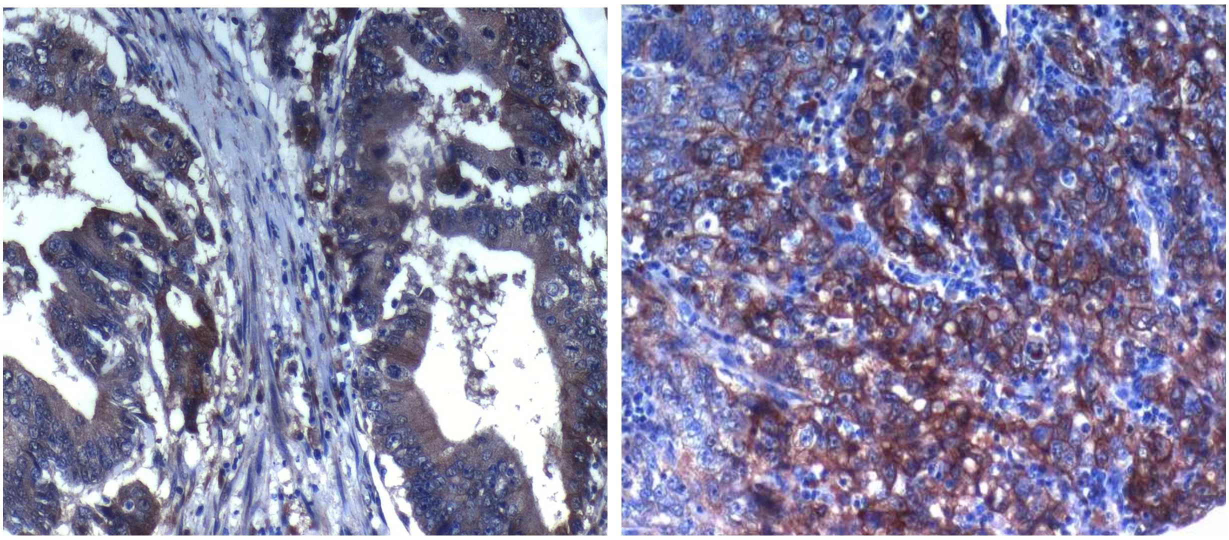


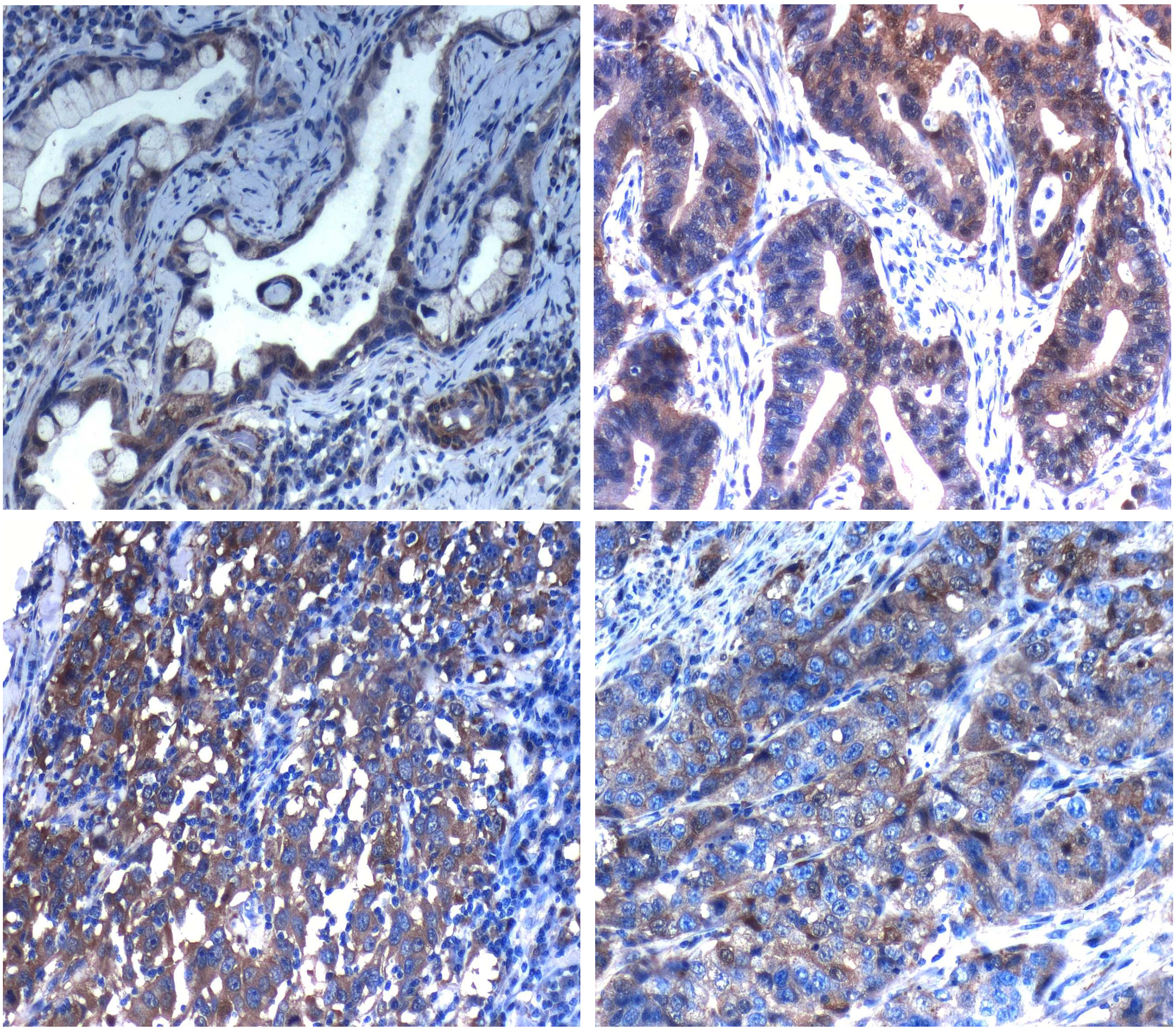


7.2.1.2 Normal esophagus (corresponding to the TMA slide of esophageal adenocarcinoma)



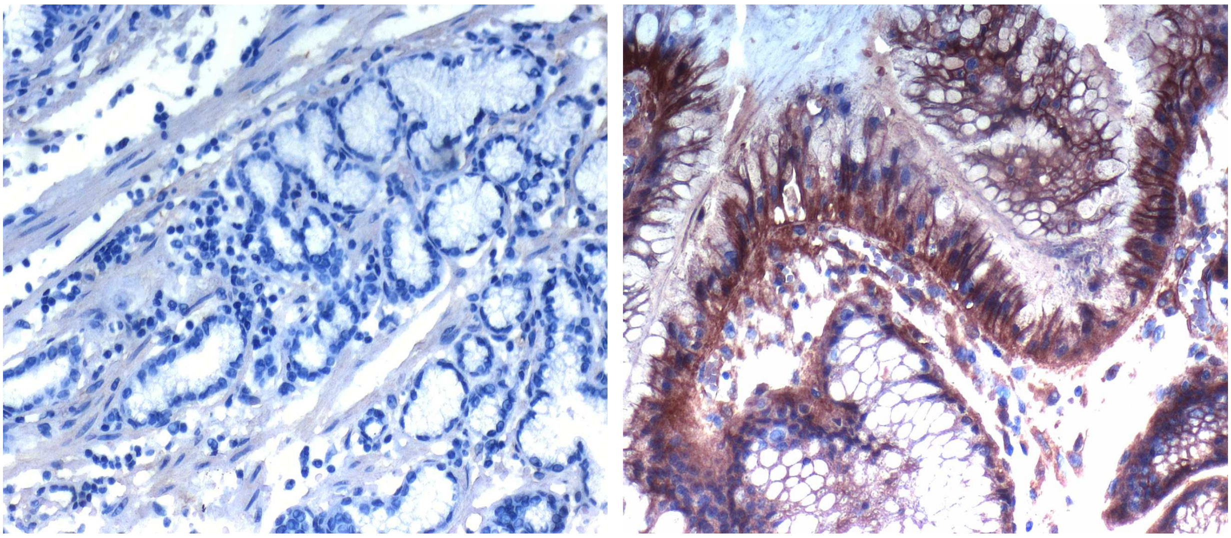
7.2.1.3 Esophageal adenocarcinoma



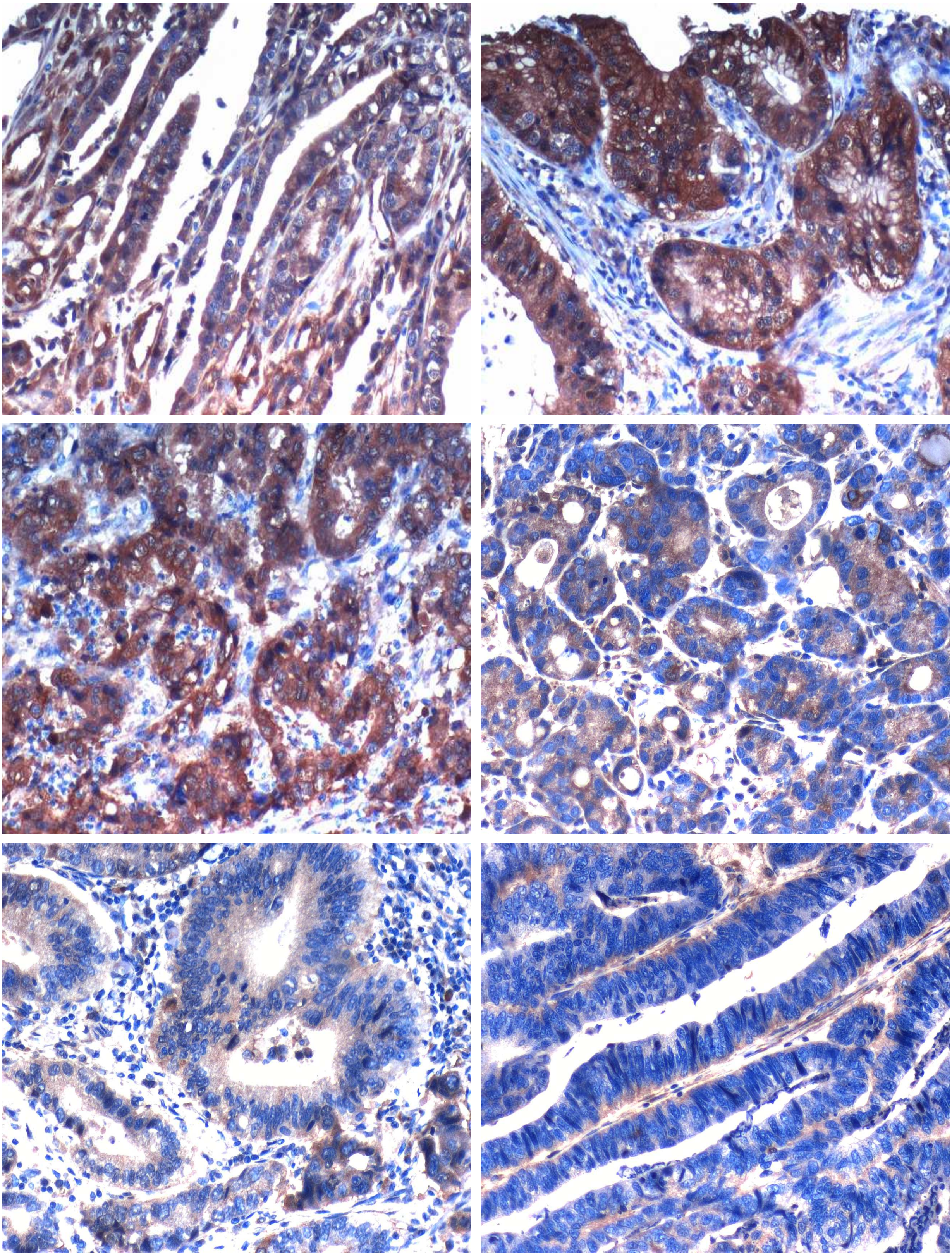


7.2.2 Cardia

7.2.2.1 Normal cardia

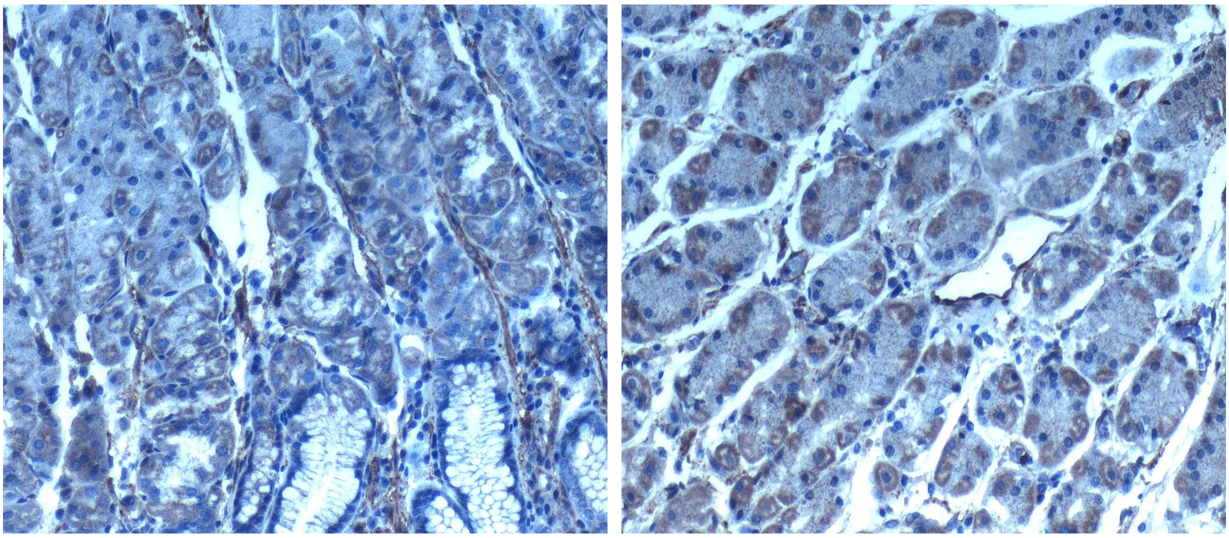


7.2.2.2 Cardia adenocarcinoma

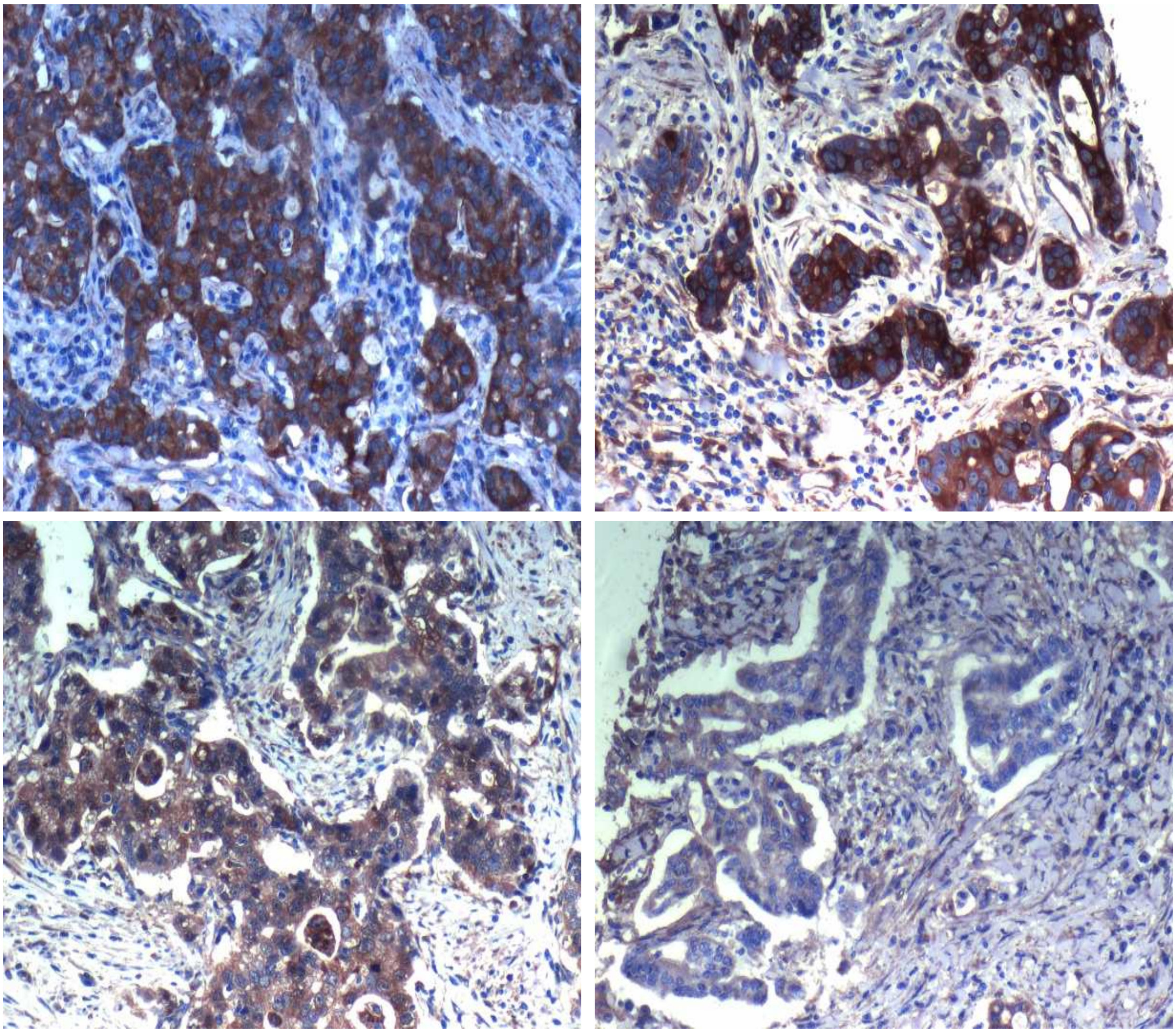


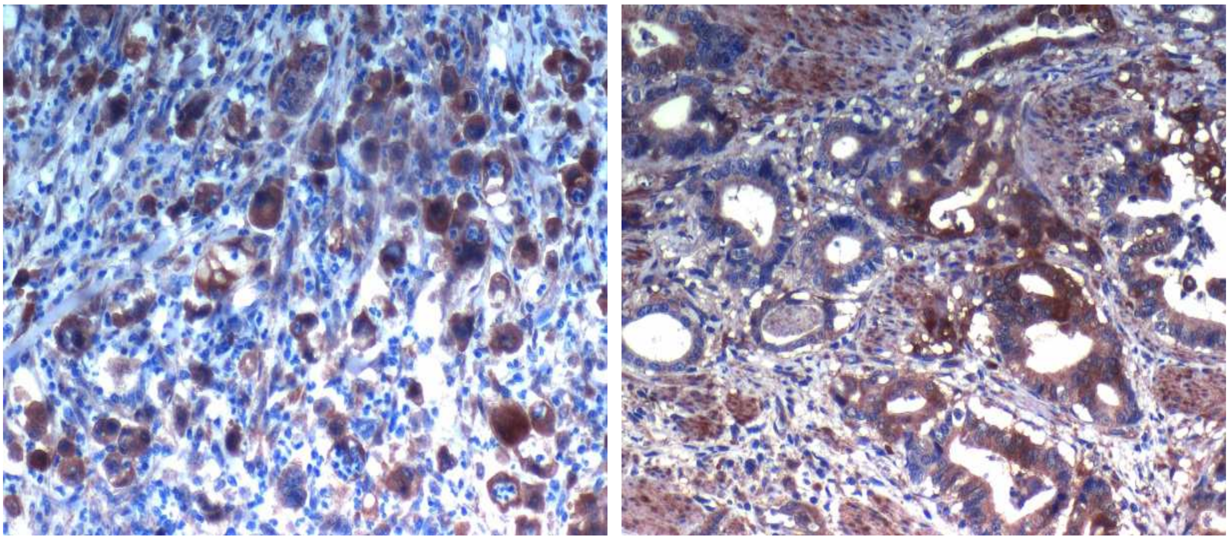
7.2.3 Stomach

7.2.3.1 Normal stomach



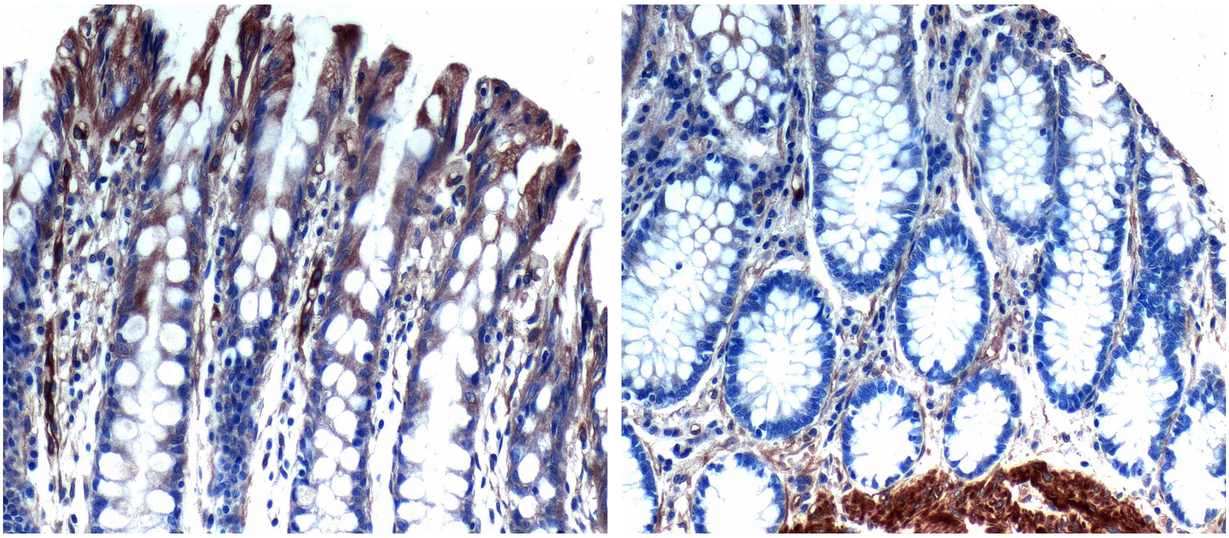
7.2.3.2 Stomach adenocarcinoma



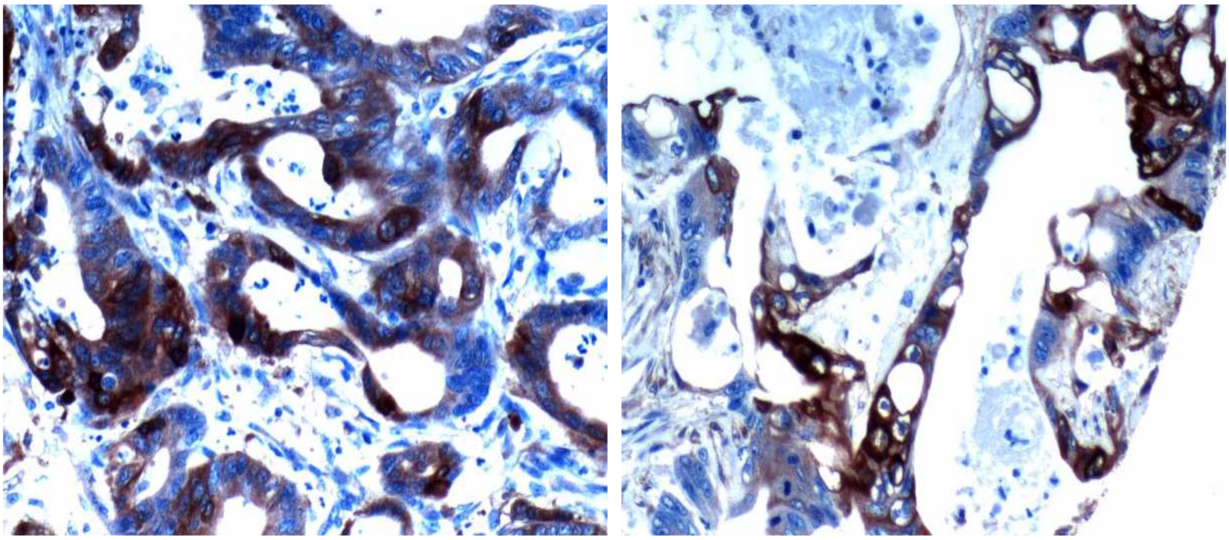


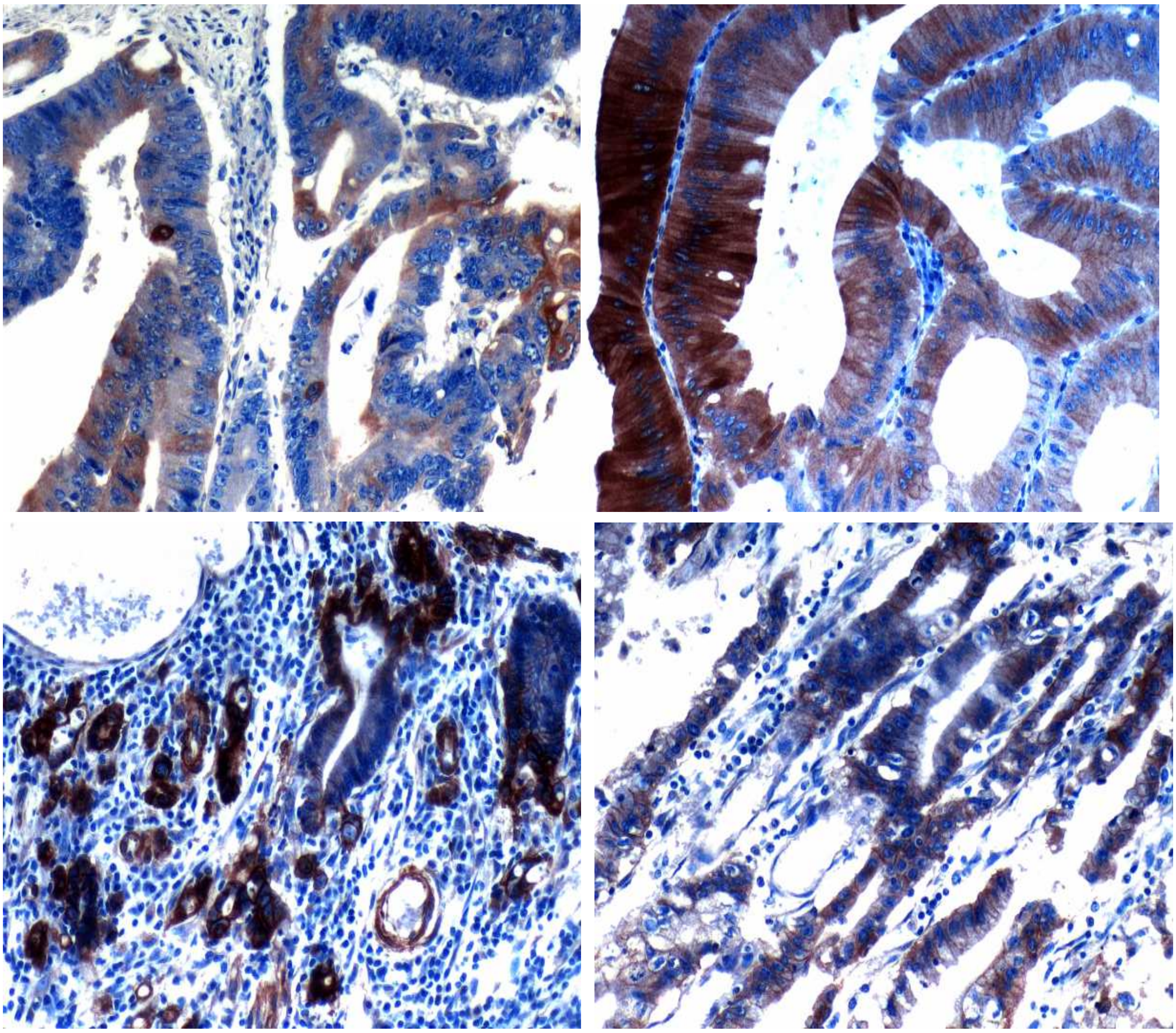
7.2.4 Colon-rectum

7.2.4.1 Normal colon-rectum



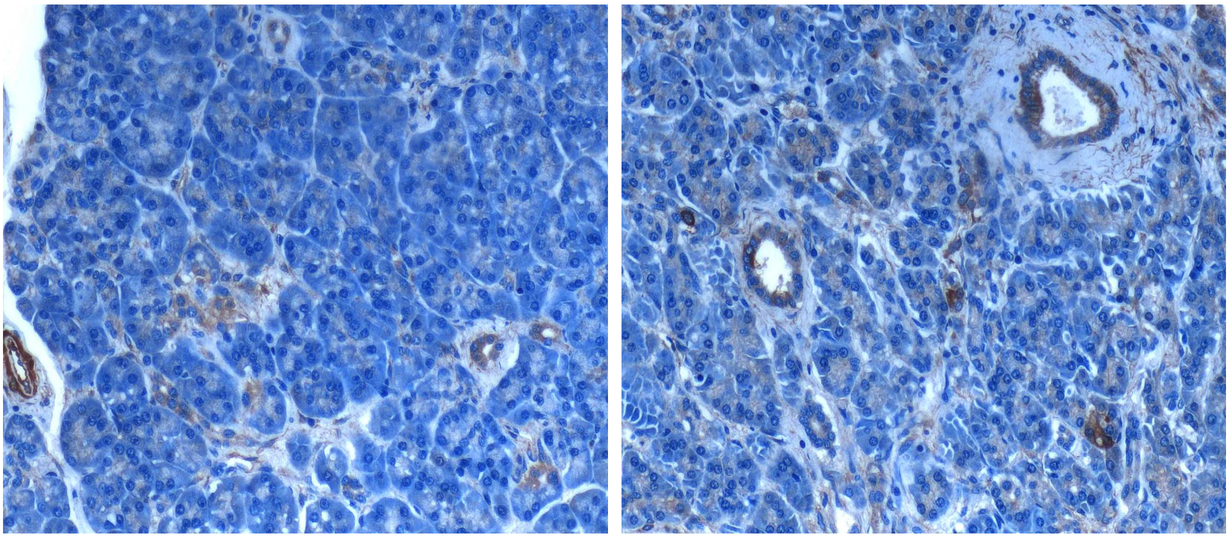
7.2.4.2 Colorectal adenocarcinoma



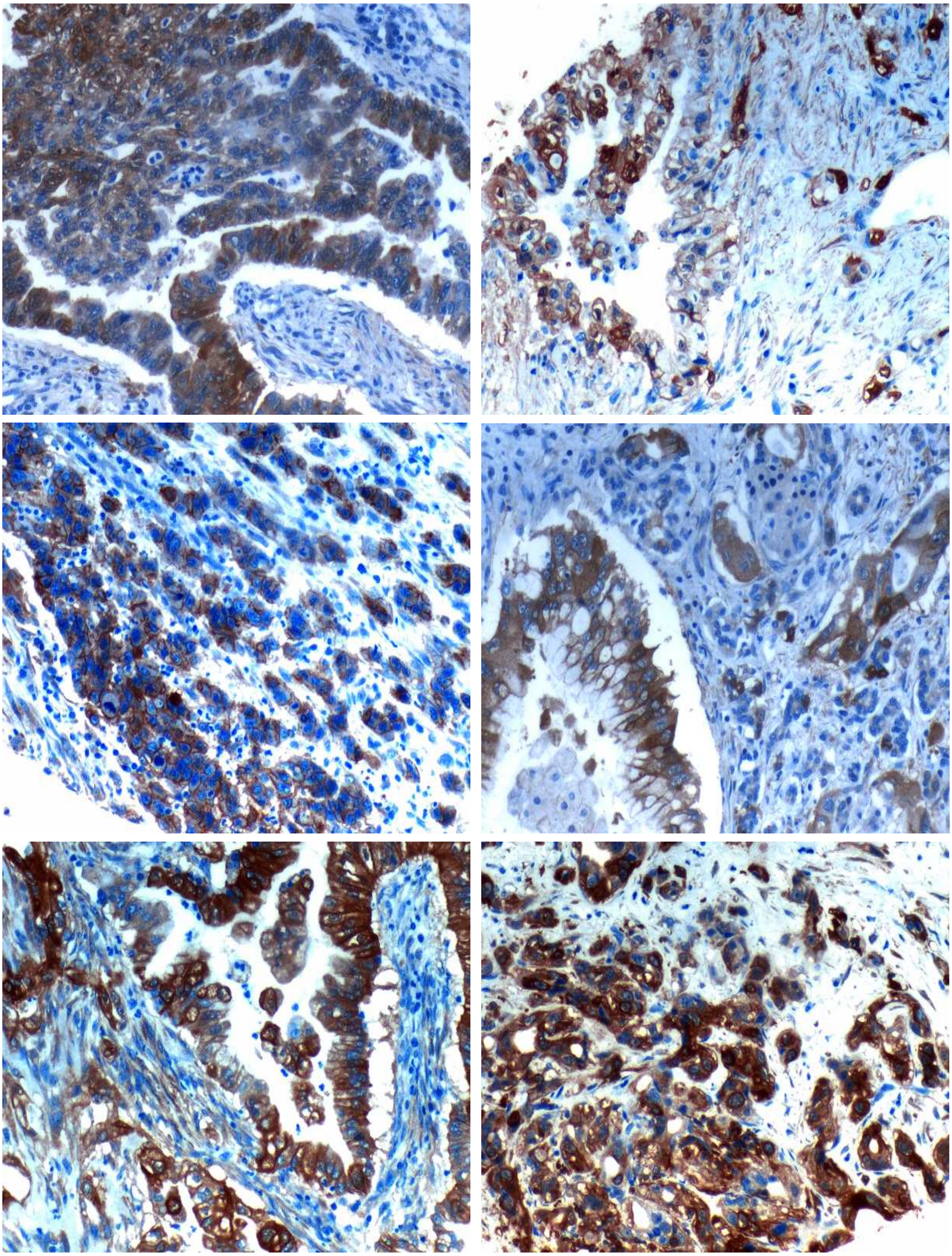


7.2.5 Pancreas

7.2.5.1 Normal pancreas

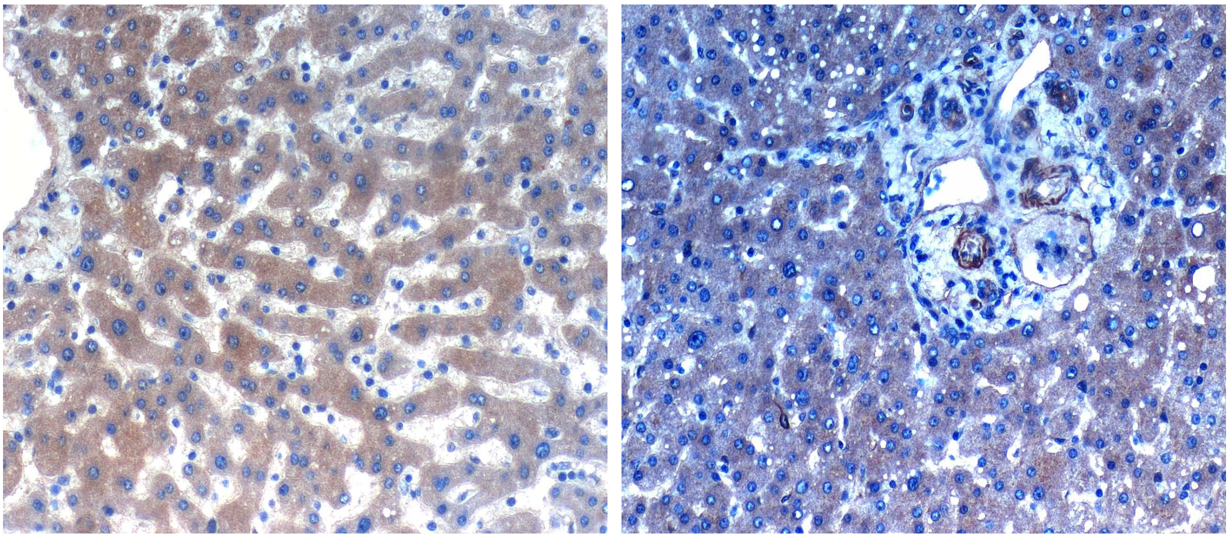


7.2.5.2 Pancreatic adenocarcinoma

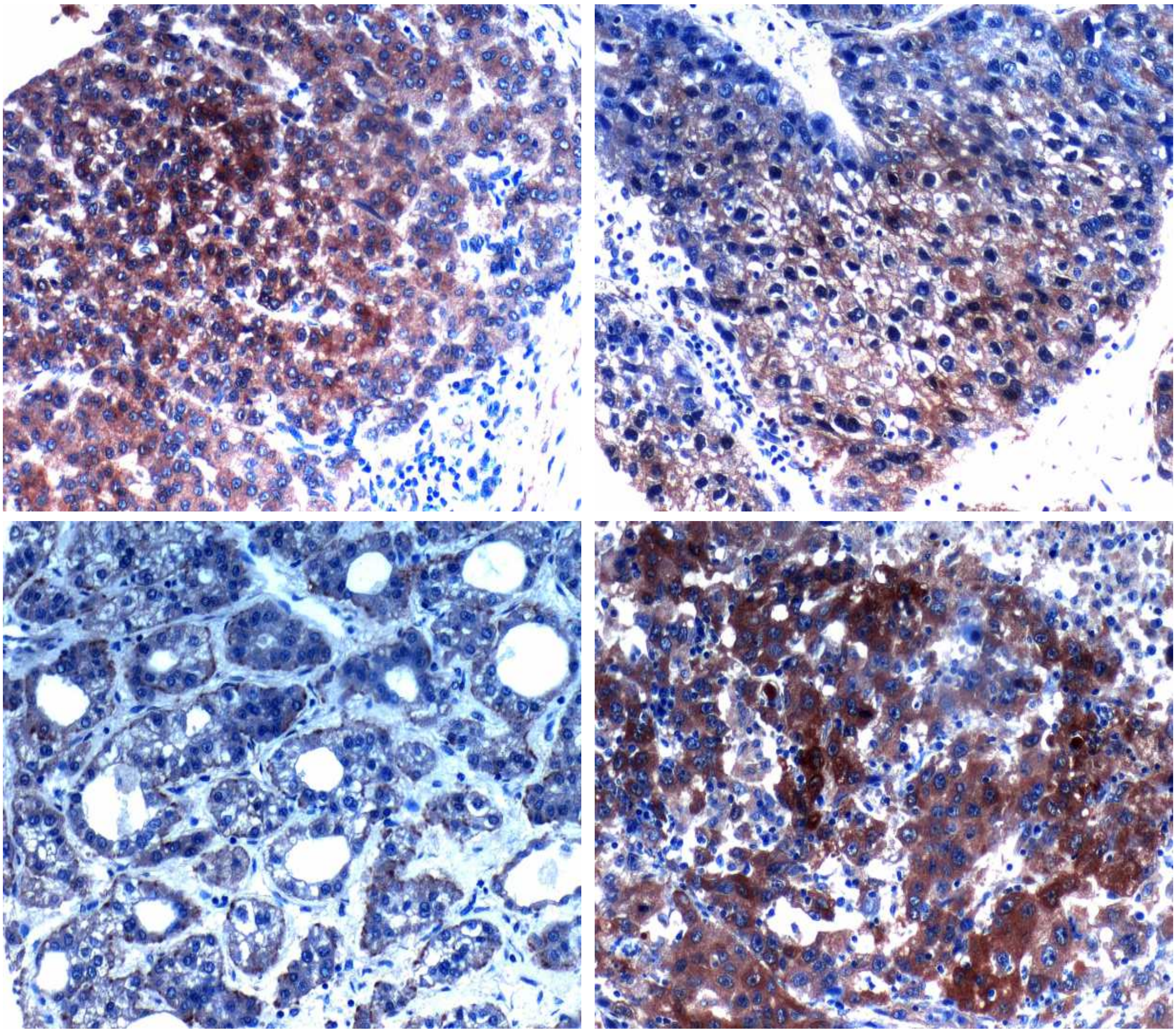


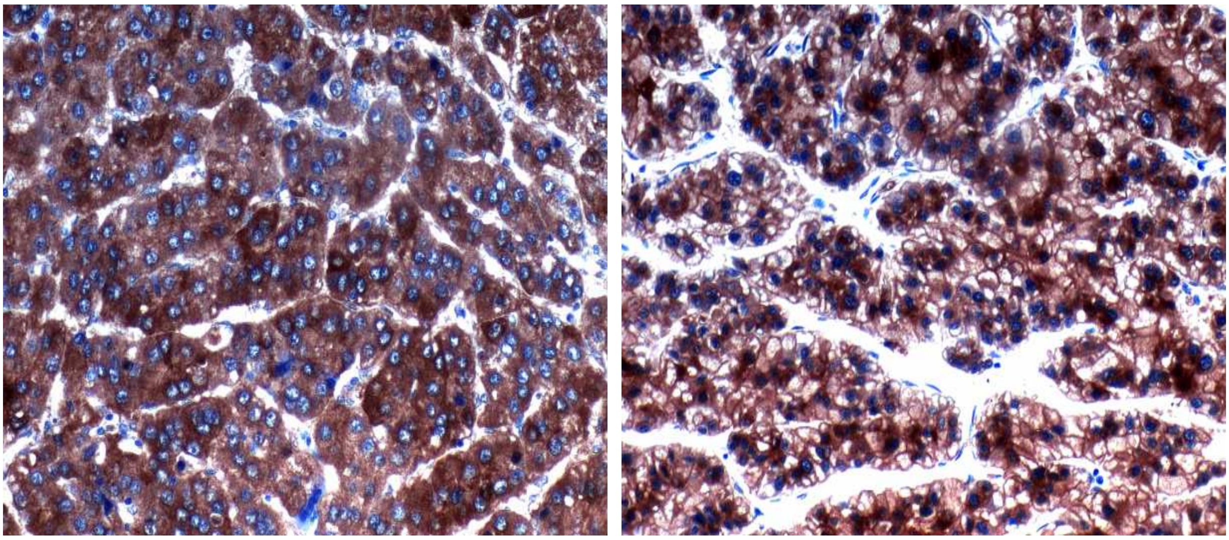
7.2.6 Liver

7.2.6.1 Normal liver

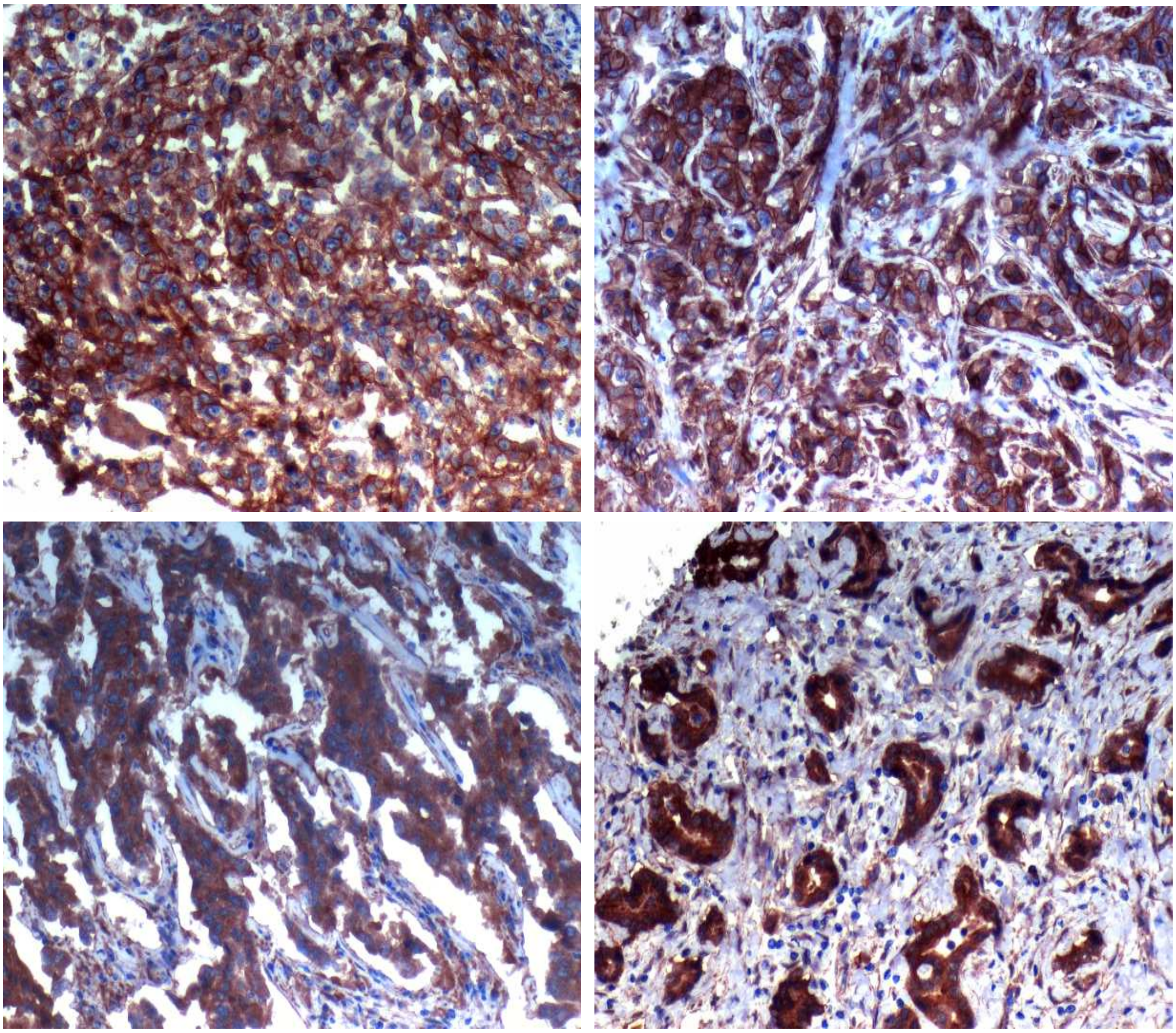


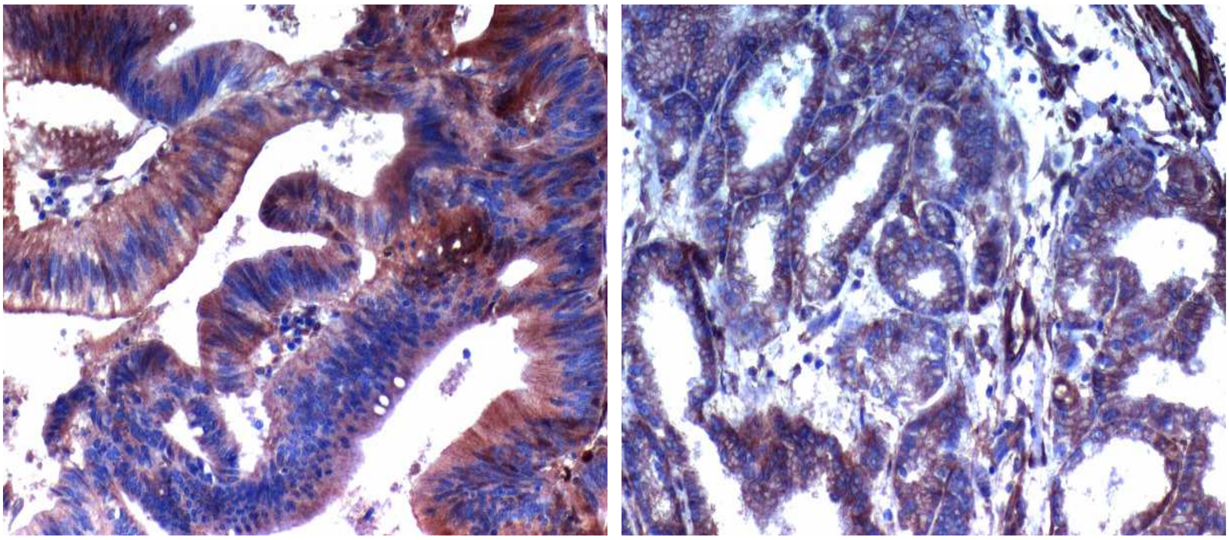
7.2.6.2 Hepatocellular carcinoma





7.2.6.3 Cholangiocarcinoma





7.3 Abbreviations

mg	Milligram
µg	Microgram
µl	Microliter
min	Minute
ml	Milliliter
mM	Millimole
M	Mole
µm	Micrometer
nm	Nanometer
sec	Second
V	Volt
kDa	Kilodalton
rpm	Revolutions per minute
APS	Ammonium persulfate
Akt	Protein Kinase B
BL22	Anti-CD22 immunotoxin fusion protein (murine anti-CD22 disulfide-linked Fv (dsFv) antibody fragment was fused to an edited copy of bacterial Pseudomonas exotoxin PE38)
BSA	Bovine serum albumin
CD20	Cluster of differentiation protein-20
CD22	Cluster of differentiation protein -22
CD3	Cluster of differentiation protein -3

cDNA	Complementary DNA
DAB	3,3'-Diaminobenzidine
Erk	Extracellular-signal-regulated kinase
ERBB2	Human epidermal factor receptor 2
Fak	Focal Adhesion Kinase
HER2	Human epidermal factor receptor 2
H-Ras	GTPase Hras
H2O2	Hydrogen peroxide
IgG	Immunoglobulin G
IHC	Immunohistochemistry
Mek1	Mitogen-activated protein kinase-1
PDZ	An acronym combining the first letters of three proteins: post synaptic density protein (PSD95), drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PBS	Phosphate buffered saline
Rac1	Ras-related C3 botulinum toxin substrate 1
RHOA	Ras homolog gene family, member A
Src	Proto-oncogene tyrosine-protein kinase
SDS	Sodium dodecyl sulfate
TBST	TBS with Tween 20
Tris	Tris(hydroxymethyl)aminomethane
TEMED	N,N,N',N'-Tetramethylethylenediamine
VEGF-A	Vascular endothelial growth factor-A

7.4 Figure index

7.5 Table index

8. Eidesstattliche Versicherung

„Ich, Jing Du, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Expression patterns of EphA2 tyrosine kinase receptor in human digestive system organs and tumors selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.

Datum 25.07.2013 Unterschrift Jing Du

9. Curriculum Vitae

Due to privacy reasons, my CV is not published in the e-version of the thesis.

10. Publication

None of this work has been published so far.

11. Acknowledgments

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